ENTERICALLY TRANSMITTED NON-A/NON-B HEPATITIS VIRAL AGENT AND CHARACTERISTIC EPITOPES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Application Serial No. 08/279,823, filed July 25, 1994, which is a continuation of U.S. Application Serial No. 07/681,078, filed April 5, 1991, now abandoned, which is a continuation-in-part of U.S. Application Serial No.07/505,888, filed April 5, 1990, now abandoned, which is a continuation-in-part of U.S. Application Serial No. 07/420,921, filed October 13, 1989, now abandoned, which is a continuation-in-part of U.S. Application Serial No. 07/367,486, filed June 16, 1989, now abandoned, which is a continuation-in-part of U.S. Application Serial No. 07/336,672, filed April 11, 1989, now abandoned, which is a continuation-in-part of U.S. Application Serial No. 07/208,997, filed June 17, 1988, now abandoned, all of which are herein incorporated by reference.

INTRODUCTION

Field of Invention

This invention relates to recombinant proteins, genes, and gene probes and more specifically to such proteins and probes derived from an enterically transmitted nonA/nonB hepatitis viral agent, to diagnostic methods and vaccine applications which employ the proteins and probes, and to gene segments that encode specific epitopes (and proteins artificially produced to contain those epitopes) that are particularly useful in diagnosis and prophylaxis.

Background

Enterically transmitted non-A/non-B hepatitis viral agent (ET-NANB; also referred to herein as HEV) is the reported cause of hepatitis in several epidemics and sporadic cases in Asia, Africa, Europe, Mexico, and the Indian subcontinent. Infection is usually by water contaminated with feces, although

the virus may also spread by close physical contact. The virus does not seem to cause chronic infection. The viral etiology in ET-NANB has been demonstrated by infection of volunteers with pooled fecal isolates; immune electron microscopy (IEM) studies have shown virus particles with 27-34 nm diameters in stools from infected individuals. The virus particles reacted with antibodies in serum from infected individuals from geographically distinct regions, suggesting that a single viral agent or class is responsible for the majority of ET-NANB hepatitis seen worldwide. No antibody reaction was seen in serum from individuals infected with parenterally transmitted NANB virus (also known as hepatitis C virus or HCV), indicating a different specificity between the two NANB types.

In addition to serological differences, the two types of NANB infection show distinct clinical differences. ET-NANB is characteristically an acute infection, often associated with fever and arthralgia, and with portal inflammation and associated bile stasis in liver biopsy specimens (Arankalle). Symptoms are usually resolved within six weeks. Parenterally transmitted NANB, by contrast, produces a chronic infection in about 50% of the cases. Fever and arthralgia are rarely seen, and inflammation has a predominantly parenchymal distribution (Khuroo, 1980). The course of ET-NANBH is generally uneventful in healthy individuals, and the vast majority of those infected recover without the chronic sequelae seen with HCV. One peculiar epidemiologic feature of this disease, however, is the markedly high mortality observed in pregnant women; this is reported in numerous studies to be on the order of 10-20%. This finding has been seen in a number of epidemiologic studies but at present remains unexplained. Whether this reflects viral pathogenicity, the lethal consequence of the interaction of virus and immune suppressed (pregnant) host, or a reflection of the

debilitated prenatal health of a susceptible malnourished population remains to be clarified.

The two viral agents can also be distinguished on the basis of primate host susceptibility. ET-NANB, but not the parenterally transmitted agent, can be transmitted to cynomolgus monkeys. The parenterally transmitted agent is more readily transmitted to chimpanzees than is ET-NANB (Bradley, 1987).

There have been major efforts worldwide to identify and clone viral genomic sequences associated with ET-NANB hepatitis. One goal of this effort, requiring virus-specific genomic sequences, is to identify and characterize the nature of the virus and its protein products. Another goal is to produce recombinant viral proteins which can be used in antibody-based diagnostic procedures and for a vaccine. Despite these efforts, viral sequences associated with ET-NANB hepatitis have not been successfully identified or cloned heretofore, nor have any virus-specific proteins been identified or produced.

Relevant Literature

Arankalle, V.A., et al., The Lancet, 550 (March 12, 1988).

Bradley, D.W., et al., J Gen. Virol., 69:1 (1988).

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Gravelle, C.R. et al., J. Infect. Diseases, 131:167 (1975).

Kane, M.A., et al., JAMA, 252:3140 (1984).

Khuroo, M.S., Am. J. Med., 48:818 (1980).

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Maniatis, T., et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1982).

Seto, B., et al., Lancet, 11:941 (1984).

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SUMMARY OF THE INVENTION

Novel compositions, as well as methods of preparation and use of the compositions are provided, where the compositions comprise viral proteins and fragments thereof derived from the viral agent for ET-NANB. A number of specific fragments of viral proteins (and the corresponding genetic sequences) that are particularly useful in diagnosis and vaccine production are also disclosed. Methods for preparation of ET-NANB viral proteins include isolating ET-NANB genomic sequences which are then cloned and expressed in a host cell. The resultant recombinant viral proteins find use as diagnostic agents and as vaccines. The genomic sequences and fragments thereof find use in preparing ET-NANB viral proteins and as probes for virus detection.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows vector constructions and manipulations used in obtaining and sequencing cloned ET-NANB fragment; and

Figures 2A-2B are representations of Southern blots in which a radiolabeled ET-NANB probe was hybridized with amplified cDNA fragments prepared from RNA isolated from infected (I) and non-infected (N) bile sources (2A), and from infected (I) and non-infected (N) stool-sample sources (2B).

DESCRIPTION OF SPECIFIC EMBODIMENTS

Novel compositions comprising generic sequences and fragments thereof derived from the viral agent for ET-NANB are provided, together with recombinant viral proteins produced using the genomic sequences and methods of using these compositions. Epitopes on the viral protein have been identified that are particularly useful in diagnosis and vaccine production. Small peptides containing the epitopes are recognized by multiple sera of patients infected with ET-NANB.

The molecular cloning of HEV was accomplished by two very different approaches. The first successful identification of a molecular clone was based on the differential hybridization of putative HEV cDNA clones to heterogeneous cDNA from infected and uninfected cyno bile. cDNAs from both sources were labeled to high specific activity with ^{32}P to identify a clone that hybridized specifically to the infected source probe. A cyno monkey infected with the Burma isolate of HEV was used in these first experiments. The sensitivity of this procedure is directly related to the relative abundance of the specific sequence against the overall background. In control experiments, it was found that specific identification of a target sequence may be obtained with as little as 1 specific part per 1000 background sequences. A number of clones were identified by this procedure using libraries and probes made from infected (Burma isolate) and control uninfected cyno bile. The first extensively characterized clone of the 16 plaques purified by this protocol was given the designation ET1.1.

ET1.1 was first characterized as both derived from and unique to the infected source cDNA. Heterogeneous cDNA was amplified from both infected and uninfected sources using a sequence independent single premier amplification technique (SISPA). This

technique is described in copending application serial No. 208,512, filed June 17, 1988. The limited pool of cDNA made from Burma infected cyno bile could then be amplified enzymatically prior to cloning or hybridization using putative HEV clones as probes. ET1.1 hybridized specifically to the original bile cDNA from the infected source. Further validation of this clone as derived from the genome of HEV was demonstrated by the similarity of the ET1.1 sequence and those present in SISPA cDNA prepared from five different human stool samples collected from different ET-NANBH epidemics including Somalia, Tashkent, Borneo, Mexico and Pakistan. These molecular epidemiologic studies established the isolated sequence as derived from the virus that represented the major cause of ET-NANBH worldwide.

The viral specificity of ET1.1 was further established by the finding that the clone hybridized specifically to RNA extracted from infected cyno liver. Hybridization analysis of polyadenylated RNA demonstrated a unique 7.5 Kb polyadenylated transcript not present in uninfected liver. The size of this transcript suggested that it represented the full length viral genome. Strand specific oligonucleotides were also used to probe viral genomic RNA extracted directly from semi-purified virions prepared from human stool. The strand specificity was based on the RNA-directed RNA polymerase (RDRP) open reading frame (ORF) identified in ET1.1 (see below). Only the probe detecting the sense strand hybridized to the nucleic acid. These studies characterized HEV as a plus sense, single stranded genome. Strand specific hybridization to RNA extracted from the liver also established that the vast majority of intracellular transcript was positive sense. Barring any novel mechanism for virus expression, the negative strand, although not detectable, would be present at a

ratio of less than 1:100 when compared with the sense strand.

ET1.1 was documented as exogenous when tested by both Southern blot hybridization and PCR using genomic DNAs derived from uninfected humans, infected and uninfected cynos and also the genomic DNAs from E. coli and various bacteriophage sources. The latter were tested in order to rule out trivial contamination with an exogenous sequence introduced during the numerous enzymatic manipulations performed during cDNA construction and amplification. It was also found that the nucleotide sequence of the ET1.1 clone was not homologous to any entries in the Genebank database. The translated open reading frame of the ET1.1 clone did, however, demonstrate limited homology with consensus amino acid residues consistent with an RNA-directed RNA polymerase. This consensus amino acid motif is shared among all positive strand RNA viruses and, as noted above, is present at the 3' end of the HCV genome. The 1.3 Kb clone was therefore presumed to be derived, at least in part, from the nonstructural portion of the viral genome.

Because of the relationship of different strains of ET-NANB to each other that has been demonstrated by the present invention, the genome of the ET-NANB viral agent is defined in this specification as containing a region which is homologous to the 1.33 kb DNA EcoRI insert present in plasmid pTZKF1 (ET1.1) carried in E. coli strain BB4 and having ATCC deposit no. 67717. The entire sequence, in both directions, has now been identified as set forth below. The sequences of both strands are provided, since both strands can encode proteins. However, the sequence in one direction has been designated as the "forward" sequence because of statistical similarities to known proteins and because the forward sequence is known to be predominately protein-encoding. This sequence is set forth below

along with the three possible translation sequences. There is one long open reading frame that starts at nucleotide 145 with an isoleucine and extends to the end of the sequence. The two other reading frames have many termination codons. Standard abbreviations for nucleotides and amino acids are used here and elsewhere in this specification.

The gene sequence given below is substantially identical to one given in the parent application. The present sequence differs in the omission of the first 37 nucleotides at the 5' end and last 13 nucleotides at the 3' end, which are derived from the linker used for cloning rather than from the virus. In addition, a G was omitted at position 227 of the sequence given in the parent application.

The following gene sequence has SEQ ID NO.1; the first amino acid sequence in reading frame beginning with nucleotide 1 has SEQ ID NO.2; the second amino acid sequence in reading frame beginning with nucleotide 2 has SEQ ID NO.3; and the third amino acid sequence in reading frame beginning with nucleotide 3 has SEQ ID NO.4.

Forward Sequence

SEQ ID NO. 1:

```
AGACCTGTCC CTGTTGCAGC TGTTCTACCA CCCTGCCCCG AGCTCGAACA GGGCCTTCTC     60

TACCTGCCCC AGGAGCTCAC CACCTGTGAT AGTGTCGTAA CATTTGAATT AACAGACATT    120

GTGCACTGCC GCATGGCCGC CCCGAGCCAG CGCAAGGCCG TGCTGTCCAC ACTCGTGGGC    180

CGCTACGGCG GTCGCACAAA GCTCTACAAT GCTTCCCACT CTGATGTTCG CGACTCTCTC    240

GCCCGTTTTA TCCCGGCCAT TGGCCCCGTA CAGGTTACAA CTTGTGAATT GTACGAGCTA    300

GTGGAGGCCA TGGTCGAGAA GGGCCAGGAT GGCTCCGCCG TCCTTGAGCT TGATCTTTGC    360

AACCGTGACG TGTCCAGGAT CACCTTCTTC CAGAAAGATT GTAACAAGTT CACCACAGGT    420

GAGACCATTG CCCATGGTAA AGTGGGCCAG GGCATCTCGG CCTGGAGCAA GACCTTCTGC    480

GCCCTCTTTG GCCCTTGGTT CCGCGCTATT GAGAAGGCTA TTCTGGCCCT GCTCCCTCAG    540

GGTGTGTTTT ACGGTGATGC CTTTGATGAG ACCGTCTTCT CGGCGGCTGT GGCCGCAGCA    600
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[redacted]GCATCCA TGGTGTTTGA GAATGACTTT TCTGAGTTTG [redacted]CCACCCA GAATAACTTT 660

TCTCTGGGTC TAGAGTGTGC TATTATGGAG GAGTGTGGGA TGCCGCAGTG GCTCATCCGC 720

CTGTATCACC TTATAAGGTC TGCGTGGATC TTGCAGGCCC CGAAGGAGTC TCTGCGAGGG 780

TTTTGGAAGA AACACTCCGG TGAGCCCGGC ACTCTTCTAT GGAATACTGT CTGGAATATG 840

GCCGTTATTA CCCACTGTTA TGACTTCCGC GATTTTCAGG TGGCTGCCTT TAAAGGTGAT 900

GATTCGATAG TGCTTTGCAG TGAGTATCGT CAGAGTCCAG GAGCTGCTGT CCTGATCGCC 960

GGCTGTGGCT TGAAGTTGAA GGTAGATTTC CGCCCGATCG GTTTGTATGC AGGTGTTGTG 1020

GTGGCCCCCG GCCTTGGCGC GCTCCCTGAT GTTGTGCGCT TCGCCGGCCG GCTTACCGAG 1080

AAGAATTGGG GCCCTGGCCC TGAGCGGGCG GAGCAGCTCC GCCTCGCTGT TAGTGATTTC 1140

CTCCGCAAGC TCACGAATGT AGCTCAGATG TGTGTGGATG TTGTTTCCCG TGTTTATGGG 1200

GTTTCCCCTG GACTCGTTCA TAACCTGATT GGCATGCTAC AGGCTGTTGC TGATGGCAAG 1260

GCACATTTCA CTGAGTCAGT AAAACCAGTG CTCGA 1295

SEQ ID NO. 2:

Arg Pro Val Pro Val Ala Ala Val Leu Pro Pro Cys Pro Glu Leu Glu
1 5 10 15

Gln Gly Leu Leu Tyr Leu Pro Gln Glu Leu Thr Thr Cys Asp Ser Val
20 25 30

Val Thr Phe Glu Leu Thr Asp Ile Val His Cys Arg Met Ala Ala Pro
35 40 45

Ser Gln Arg Lys Ala Val Leu Ser Thr Leu Val Gly Arg Tyr Gly Gly
50 55 60

Arg Thr Lys Leu Tyr Asn Ala Ser His Ser Asp Val Arg Asp Ser Leu
65 70 75 80

Ala Arg Phe Ile Pro Ala Ile Gly Pro Val Gln Val Thr Thr Cys Glu
85 90 95

Leu Tyr Glu Leu Val Glu Ala Met Val Glu Lys Gly Gln Asp Gly Ser
100 105 110

Ala Val Leu Glu Leu Asp Leu Cys Asn Arg Asp Val Ser Arg Ile Thr
115 120 125

Phe Phe Gln Lys Asp Cys Asn Lys Phe Thr Thr Gly Glu Thr Ile Ala
130 135 140

His Gly Lys Val Gly Gln Gly Ile Ser Ala Trp Ser Lys Thr Phe Cys
145 150 155 160

Leu Phe Gly Pro Trp Phe Arg Ala Ile Glu Gly Ala Ile Leu Ala
165 170 175

Leu Leu Pro Gln Gly Val Phe Tyr Gly Asp Ala Phe Asp Asp Thr Val
180 185 190

Phe Ser Ala Ala Val Ala Ala Ala Lys Ala Ser Met Val Phe Glu Asn
195 200 205

Asp Phe Ser Glu Phe Asp Ser Thr Gln Asn Asn Phe Ser Leu Gly Leu
210 215 220

Glu Cys Ala Ile Met Glu Glu Cys Gly Met Pro Gln Trp Leu Ile Arg
225 230 235 240

Leu Tyr His Leu Ile Arg Ser Ala Trp Ile Leu Gln Ala Pro Lys Glu
245 250 255

Ser Leu Arg Gly Phe Trp Lys Lys His Ser Gly Glu Pro Gly Thr Leu
260 265 270

Leu Trp Asn Thr Val Trp Asn Met Ala Val Ile Thr His Cys Tyr Asp
275 280 285

Phe Arg Asp Phe Gln Val Ala Ala Phe Lys Gly Asp Asp Ser Ile Val
290 295 300

Leu Cys Ser Glu Tyr Arg Gln Ser Pro Gly Ala Ala Val Leu Ile Ala
305 310 315 320

Gly Cys Gly Leu Lys Leu Lys Val Asp Phe Arg Pro Ile Gly Leu Tyr
325 330 335

Ala Gly Val Val Val Ala Pro Gly Leu Gly Ala Leu Pro Asp Val Val
340 345 350

Arg Phe Ala Gly Arg Leu Thr Glu Lys Asn Trp Gly Pro Gly Pro Glu
355 360 365

Arg Ala Glu Gln Leu Arg Leu Ala Val Ser Asp Phe Leu Arg Lys Leu
370 375 380

Thr Asn Val Ala Gln Met Cys Val Asp Val Val Ser Arg Val Tyr Gly
385 390 395 400

Val Ser Pro Gly Leu Val His Asn Leu Ile Gly Met Leu Gln Ala Val
405 410 415

Ala Asp Gly Lys Ala His Phe Thr Glu Ser Val Lys Pro Val Leu
420 425 430

SEQ ID NO. 3:

Asp Leu Ser Leu Leu Gln Leu Phe Tyr His Pro Ala Pro Ser Ser Asn
1 5 10 15

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Arg Ala Phe Ser Thr Cys Pro Arg Ser Ser Pro Pro Val Ile Val Ser
            20                  25                  30

 .  His Leu Asn  .  Gln Thr Leu Cys Thr Ala Ala Trp Pro Pro Arg
 5          35                  40                  45

Ala Ser Ala Arg Pro Cys Cys Pro His Ser Trp Ala Ala Thr Ala Val
     50                  55                  60

10 Ala Gln Ser Ser Thr Met Leu Pro Thr Leu Met Phe Ala Thr Leu Ser
 65                  70                  75                  80

Pro Val Leu Ser Arg Pro Leu Ala Pro Tyr Arg Leu Gln Leu Val Asn
                85                  90                  95
15
Cys Thr Ser  .  Trp Arg Pro Trp Ser Arg Arg Ala Arg Met Ala Pro
            100                 105                 110

Pro Ser Leu Ser Leu Ile Phe Ala Thr Val Thr Cys Pro Gly Ser Pro
20          115                 120                 125

Ser Ser Arg Lys Ile Val Thr Ser Ser Pro Gln Val Arg Pro Leu Pro
    130                 135                 140

25 Met Val Lys Trp Ala Arg Ala Ser Arg Pro Gly Ala Arg Pro Ser Ala
145                 150                 155                 160

Pro Ser Leu Ala Leu Gly Ser Ala Leu Leu Arg Arg Leu Phe Trp Pro
                165                 170                 175
30
Cys Ser Leu Arg Val Cys Phe Thr Val Met Pro Leu Met Thr Pro Ser
            180                 185                 190

Ser Arg Arg Leu Trp Pro Gln Gln Arg His Pro Trp Cys Leu Arg Met
35          195                 200                 205

Thr Phe Leu Ser Leu Thr Pro Pro Arg Ile Thr Phe Leu Trp Val  .
    210                 215                 220

40 Ser Val Leu Leu Trp Arg Ser Val Gly Cys Arg Ser Gly Ser Ser Ala
225                 230                 235                 240

Cys Ile Thr Leu  .  Gly Leu Arg Gly Ser Cys Arg Pro Arg Arg Ser
                245                 250                 255
45
Leu Cys Glu Gly Phe Gly Arg Asn Thr Pro Val Ser Pro Ala Leu Phe
            260                 265                 270

Tyr Gly Ile Leu Ser Gly Ile Trp Pro Leu Leu Pro Thr Val Met Thr
50          275                 280                 285

Ser Ala Ile Phe Arg Trp Leu Pro Leu Lys Val Met Ile Arg  .  Cys
    290                 295                 300

55 Phe Ala Val Ser Ile Val Arg Val Gln Glu Leu Leu Ser  .  Ser Pro
305                 310                 315                 320
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Ala Val Ala . Ser . Arg . Ile Ser Ala Arg Ser Val Cys Met
325 330 335

Gln Val Leu Trp Trp Pro Pro Ala Leu Ala Arg Ser Leu Met Leu Cys
340 345 350

Ala Ser Pro Ala Gly Leu Pro Arg Arg Ile Gly Ala Leu Ala Leu Ser
355 360 365

Gly Arg Ser Ser Ser Ala Ser Leu Leu Val Ile Ser Ser Ala Ser Ser
370 375 380

Arg Met . Leu Arg Cys Val Trp Met Leu Phe Pro Val Phe Met Gly
385 390 395 400

Phe Pro Leu Asp Ser Phe Ile Thr . Leu Ala Cys Tyr Arg Leu Leu
405 410 415

Leu Met Ala Arg His Ile Ser Leu Ser Gln . Asn Gln Cys Ser
420 425 430

SEQ ID NO. 4:

Thr Cys Pro Cys Cys Ser Cys Ser Thr Thr Leu Pro Arg Ala Arg Thr
1 5 10 15

Gly Pro Ser Leu Pro Ala Pro Gly Ala His His Leu . . Cys Arg
20 25 30

Asn Ile . Ile Asn Arg His Cys Ala Leu Pro His Gly Arg Pro Glu
35 40 45

Pro Ala Gln Gly Arg Ala Val His Thr Arg Gly Pro Leu Arg Arg Ser
50 55 60

His Lys Ala Leu Gln Cys Phe Pro Leu . Cys Ser Arg Leu Ser Arg
65 70 75 80

Pro Phe Tyr Pro Gly His Trp Pro Arg Thr Gly Tyr Asn Leu . Ile
85 90 95

Val Arg Ala Ser Gly Gly His Gly Arg Glu Gly Pro Gly Trp Leu Arg
100 105 110

Arg Pro . Ala . Ser Leu Gln Pro . Arg Val Gln Asp His Leu
115 120 125

Leu Pro Glu Arg Leu . Gln Val His His Arg . Asp His Cys Pro
130 135 140

Trp . Ser Gly Pro Gly His Leu Gly Leu Glu Gln Asp Leu Leu Arg
145 150 155 160

Pro Leu Trp Pro Leu Val Pro Arg Tyr . Glu Gly Tyr Ser Gly Pro
165 170 175

Ala Pro Ser Gly Cys Val Leu Arg . Cys Leu . . His Arg Leu
180 185 190

Leu Gly Gly Cys Gly Arg Ser Lys Gly Ile His Gly Val . Glu .
195 200 205

Leu Phe . Val . Leu His Pro Glu . Leu Phe Ser Gly Ser Arg
210 215 220

Val Cys Tyr Tyr Gly Gly Val Trp Asp Ala Ala Val Ala His Pro Pro
225 230 235 240

Val Ser Pro Tyr Lys Val Cys Val Asp Leu Ala Gly Pro Glu Gly Val
245 250 255

Ser Ala Arg Val Leu Glu Glu Thr Leu Arg . Ala Arg His Ser Ser
260 265 270

Met Glu Tyr Cys Leu Glu Tyr Gly Arg Tyr Tyr Pro Leu Leu . Leu
275 280 285

Pro Arg Phe Ser Gly Gly Cys Leu . Arg . . Phe Asp Ser Ala
290 295 300

Leu Gln . Val Ser Ser Glu Ser Arg Ser Cys Cys Pro Asp Arg Arg
305 310 315 320

Leu Trp Leu Glu Val Glu Gly Arg Phe Pro Pro Asp Arg Phe Val Cys
325 330 335

Arg Cys Cys Gly Gly Pro Arg Pro Trp Arg Ala Pro . Cys Cys Ala
340 345 350

Leu Arg Arg Pro Ala Tyr Arg Glu Glu Leu Gly Pro Trp Pro . Ala
355 360 365

Gly Gly Ala Ala Pro Pro Arg Cys . . Phe Pro Pro Gln Ala His
370 375 380

Glu Cys Ser Ser Asp Val Cys Gly Cys Cys Phe Pro Cys Leu Trp Gly
385 390 395 400

Phe Pro Trp Thr Arg Ser . Pro Asp Trp His Ala Thr Gly Cys Cys
405 410 415

. Trp Gln Gly Thr Phe His . Val Ser Lys Thr Ser Ala Arg
420 425 430

The complementary strand, referred to here as the "reverse sequence," is set forth below in the same manner as the forward sequence set forth above. Several open reading frames, shorter than the long open reading frame found in the forward sequence, can

be seen in this reverse sequence. Because of the relative brevity of the open reading frames in the reverse direction, they are probably not expressed.

The following gene sequence has SEQ ID NO.5.

Reverse Sequence

SEQ ID NO. 5:

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TCGAGCACTG GTTTTACTGA CTGAGTGAAA TGTGCCTTGC CATCAGCAAC AGCCTGTAGC      60
ATGCCAATCA GGTTATGAAC GAGTCCAGGG GAAACCCCAT AAACACGGGA AACAACATCC     120
ACACACATCT GAGCTACATT CGTGAGCTTG CGGAGGAAAT CACTAACAGC GAGGCGGAGC     180
TGCTCCGCCC GCTCAGGGCC AGGGCCCCAA TTCTTCTCGG TAAGCCGGCC GGCGAAGCGC     240
ACAACATCAG GGAGCGCGCC AAGGCCGGGG GCCACCACAA CACCTGCATA CAAACCGATC     300
GGGCGGAAAT CTACCTTCAA CTTCAAGCCA CAGCCGGCGA TCAGGACAGC AGCTCCTGGA     360
CTCTGACGAT ACTCACTGCA AAGCACTATC GAATCATCAC CTTTAAAGGC AGCCACCTGA     420
AAATCGCGGA AGTCATAACA GTGGGTAATA ACGGCCATAT TCCAGACAGT ATTCCATAGA     480
AGAGTGCCGG GCTCACCGGA GTGTTTCTTC CAAAACCCTC GCAGAGACTC CTTCGGGGCC     540
TGCAAGATCC ACGCAGACCT TATAAGGTGA TACAGGCGGA TGAGCCACTG CGGCATCCCA     600
CACTCCTCCA TAATAGCACA CTCTAGACCC AGAGAAAAGT TATTCTGGGT GGAGTCAAAC     660
TCAGAAAAGT CATTCTCAAA CACCATGGAT GCCTTTGCTG CGGCCACAGC CGCCGAGAAG     720
ACGGTGTCAT CAAAGGCATC ACCGTAAAAC ACACCCTGAG GGAGCAGGGC CAGAATAGCC     780
TTCTCAATAG CGCGGAACCA AGGGCCAAAG AGGGCGCAGA AGGTCTTGCT CCAGGCCGAG     840
ATGCCCTGGC CCACTTTACC ATGGGCAATG GTCTCACCTG TGGTGAACTT GTTACAATCT     900
TTCTGGAAGA AGGTGATCCT GGACACGTCA CGGTTGCAAA GATCAAGCTC AAGGACGGCG     960
GAGCCATCCT GGCCCTTCTC GACCATGGCC TCCACTAGCT CGTACAATTC ACAAGTTGTA    1020
ACCTGTACGG GGCCAATGGC CGGGATAAAA CGGGCGAGAG AGTCGCGAAC ATCAGAGTGG    1080
GAAGCATTGT AGAGCTTTGT GCGACCGCCG TAGCGGCCCA CGAGTGTGGA CAGCACGGCC    1140
TTGCGCTGGC TCGGGGCGGC CATGCGGCAG TGCACAATGT CTGTTAATTC AAATGTTACG    1200
ACACTATCAC AGGTGGTGAG CTCCTGGGGC AGGTAGAGAA GGCCCTGTTC GAGCTCGGGG    1260
CAGGGTGGTA GAACAGCTGC AACAGGGACA GGTCT                               1295
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Identity of this sequence with sequences in etiologic agents has been confirmed by locating a

corresponding sequence in a viral strain isolated in Burma. The Burmese isolate contains the following sequence of nucleotides (one strand and open reading frames shown). The following gene sequence has SEQ ID NO.6; the protein sequence corresponding to ORF1 has SEQ ID NO.7; ORF2 has SEQ ID NO.8; and ORF3 has SEQ ID NO.9.

SEQUENCE OF HEV (BURMA STRAIN)

-ORF1-->

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                                  M  E  A  H  Q  F  I  K  A  P  G
AGGCAGACCACATATGTGGTCGATGCCATGGAGGCCCATCAGTTTATTAAGGCTCCTGGC

 I  T  T  A  I  E  Q  A  A  L  A  A  A  N  S  A  L  A  N  A
ATCACTACTGCTATTGAGCAGGCTGCTCTAGCAGCGGCCAACTCTGCCCTGGCGAATGCT  120

 V  V  V  R  P  F  L  S  H  Q  Q  I  E  I  L  I  N  L  M  Q
GTGGTAGTTAGGCCTTTTCTCTCTCACCAGCAGATTGAGATCCTCATTAACCTAATGCAA

 P  R  Q  L  V  F  R  P  E  V  F  W  N  H  P  I  Q  R  V  I
CCTCGCCAGCTTGTTTTCCGCCCCGAGGTTTTCTGGAATCATCCCATCCAGCGTGTCATC  240

 H  N  E  L  E  L  Y  C  R  A  R  S  G  R  C  L  E  I  G  A
CATAACGAGCTGGAGCTTTACTGCCGCGCCCGCTCCGGCCGCTGTCTTGAAATTGGCGCC

 H  P  R  S  I  N  D  N  P  N  V  V  H  R  C  F  L  R  P  V
CATCCCCGCTCAATAAATGATAATCCTAATGTGGTCCACCGCTGCTTCCTCCGCCCTGTT  360

 G  R  D  V  Q  R  W  Y  T  A  P  T  R  G  P  A  A  N  C  R
GGGCGTGATGTTCAGCGCTGGTATACTGCTCCCACTCGCGGGCCGGCTGCTAATTGCCGG

 R  S  A  L  R  G  L  P  A  A  D  R  T  Y  C  L  D  G  F  S
CGTTCCGCGCTGCGCGGGCTTCCCGCTGCTGACCGCACTTACTGCCTCGACGGGTTTTCT  480

 G  C  N  F  P  A  E  T  G  I  A  L  Y  S  L  H  D  M  S  P
GGCTGTAACTTTCCCGCCGAGACTGGCATCGCCCTCTACTCCCTTCATGATATGTCACCA

 S  D  V  A  E  A  M  F  R  H  G  M  T  R  L  Y  A  A  L  H
TCTGATGTCGCCGAGGCCATGTTCCGCCATGGTATGACGCGGCTCTATGCCGCCCTCCAT  600

 L  P  P  E  V  L  L  P  P  G  T  Y  R  T  A  S  Y  L  L  I
CTTCCGCCTGAGGTCCTGCTGCCCCCTGGCACATATCGCACCGCATCGTATTTGCTAATT

 H  D  G  R  R  V  V  V  T  Y  E  G  D  T  S  A  G  Y  N  H
CATGACGGTAGGCGCGTTGTGGTGACGTATGAGGGTGATACTAGTGCTGGTTACAACCAC  720

 D  V  S  N  L  R  S  W  I  R  T  T  K  V  T  G  D  H  P  L
GATGTCTCCAACTTGCGCTCCTGGATTAGAACCACCAAGGTTACCGGAGACCATCCCCTC

 V  I  E  R  V  R  A  I  G  C  H  F  V  L  L  L  T  A  A  P
GTTATCGAGCGGGTTAGGGCCATTGGCTGCCACTTTGTTCTCTTGCTCACGGCAGCCCCG  840

 E  P  S  P  M  P  Y  V  P  Y  P  R  S  T  E  V  Y  V  R  S
GAGCCATCACCTATGCCTTATGTTCCTTACCCCCGGTCTACCGAGGTCTATGTCCGATCG
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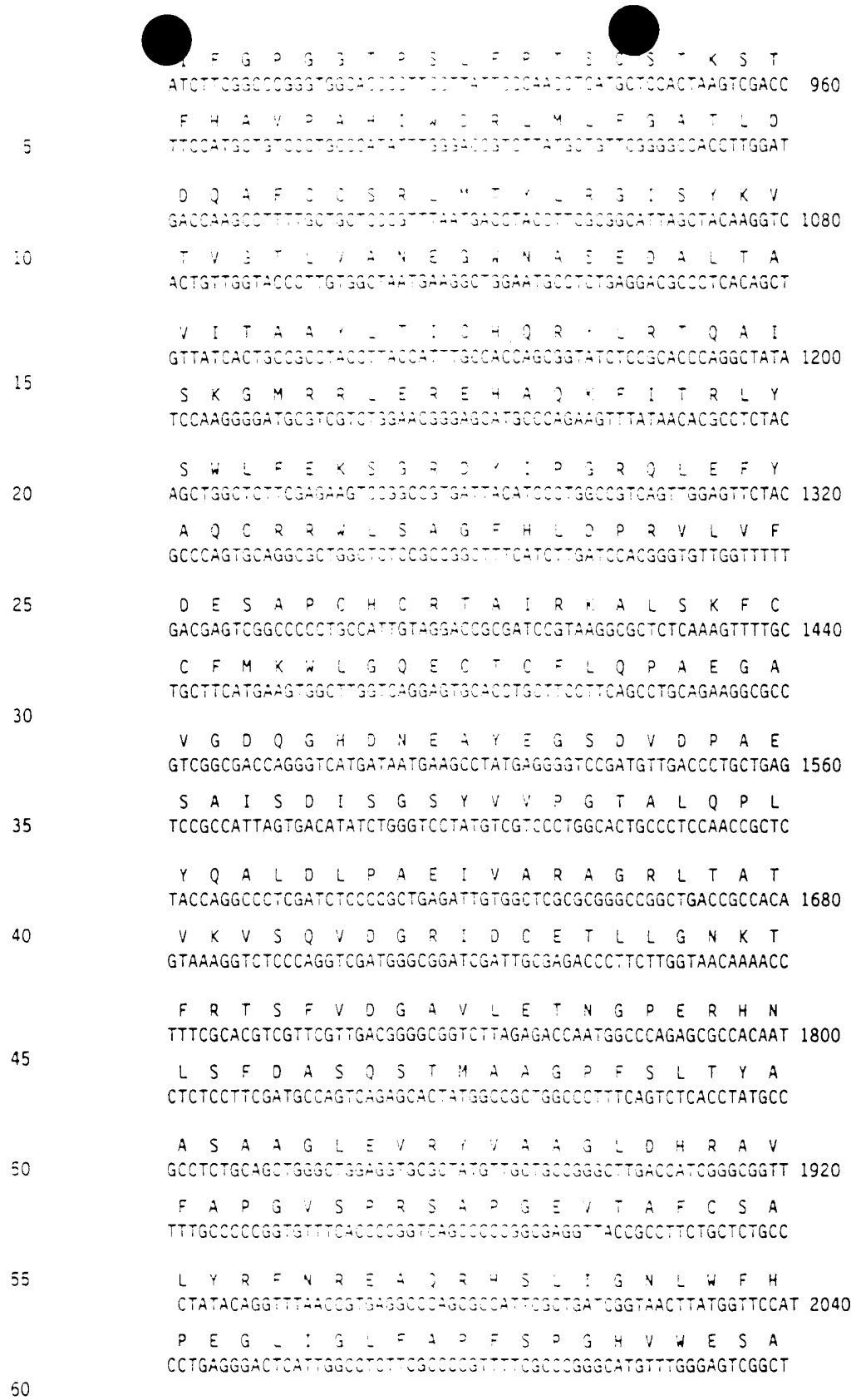
F G P G G T P S L F P T S S T K S T
ATCTTCGGCCCGGGTGGCACCGCTTCGTTATTCCCAAGCTCATGCTCCACTAAGTCGACC 960
F H A V P A H I W D R L M L F G A T L D
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D Q A F C C S R L M T Y L R G I S Y K V
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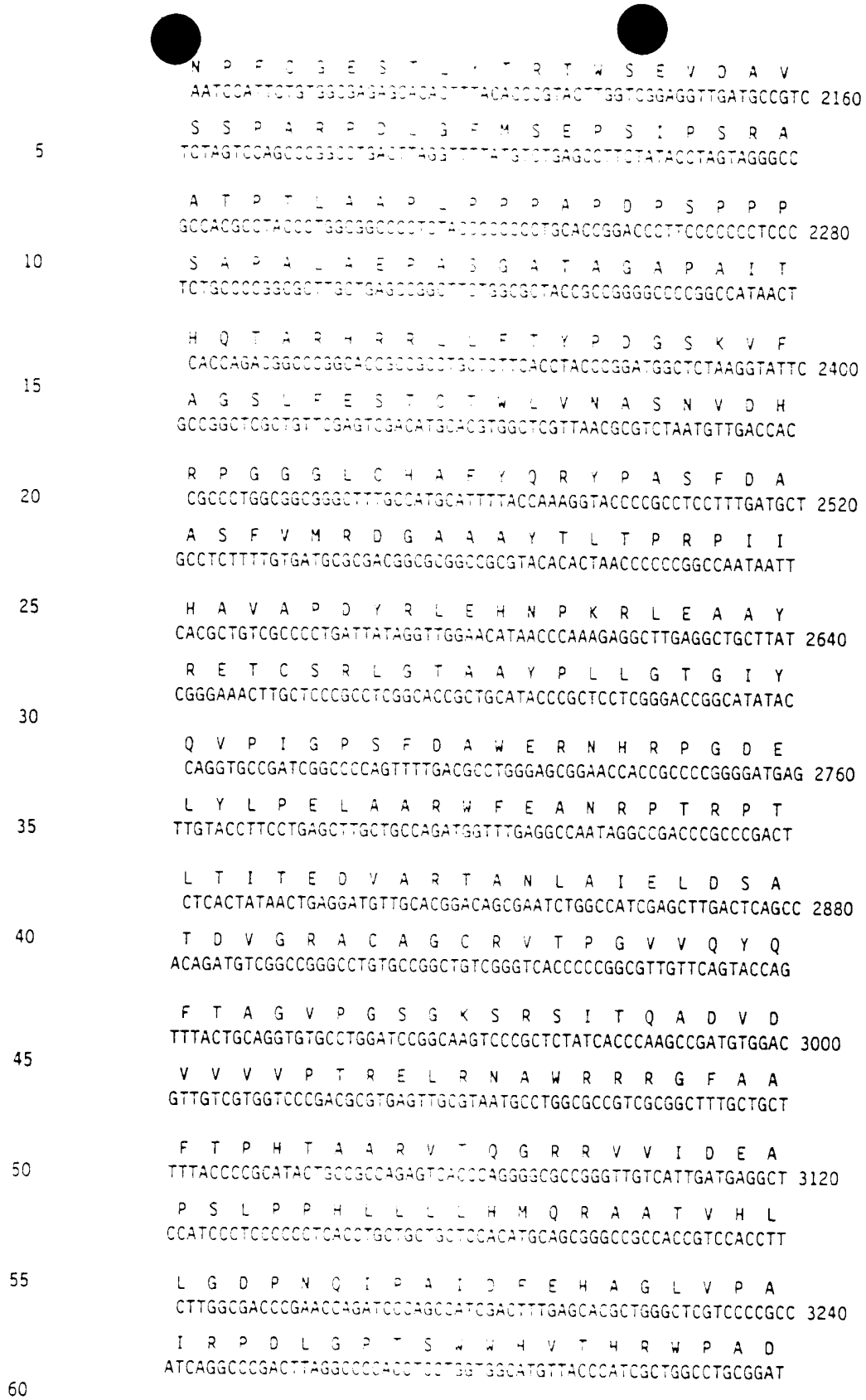
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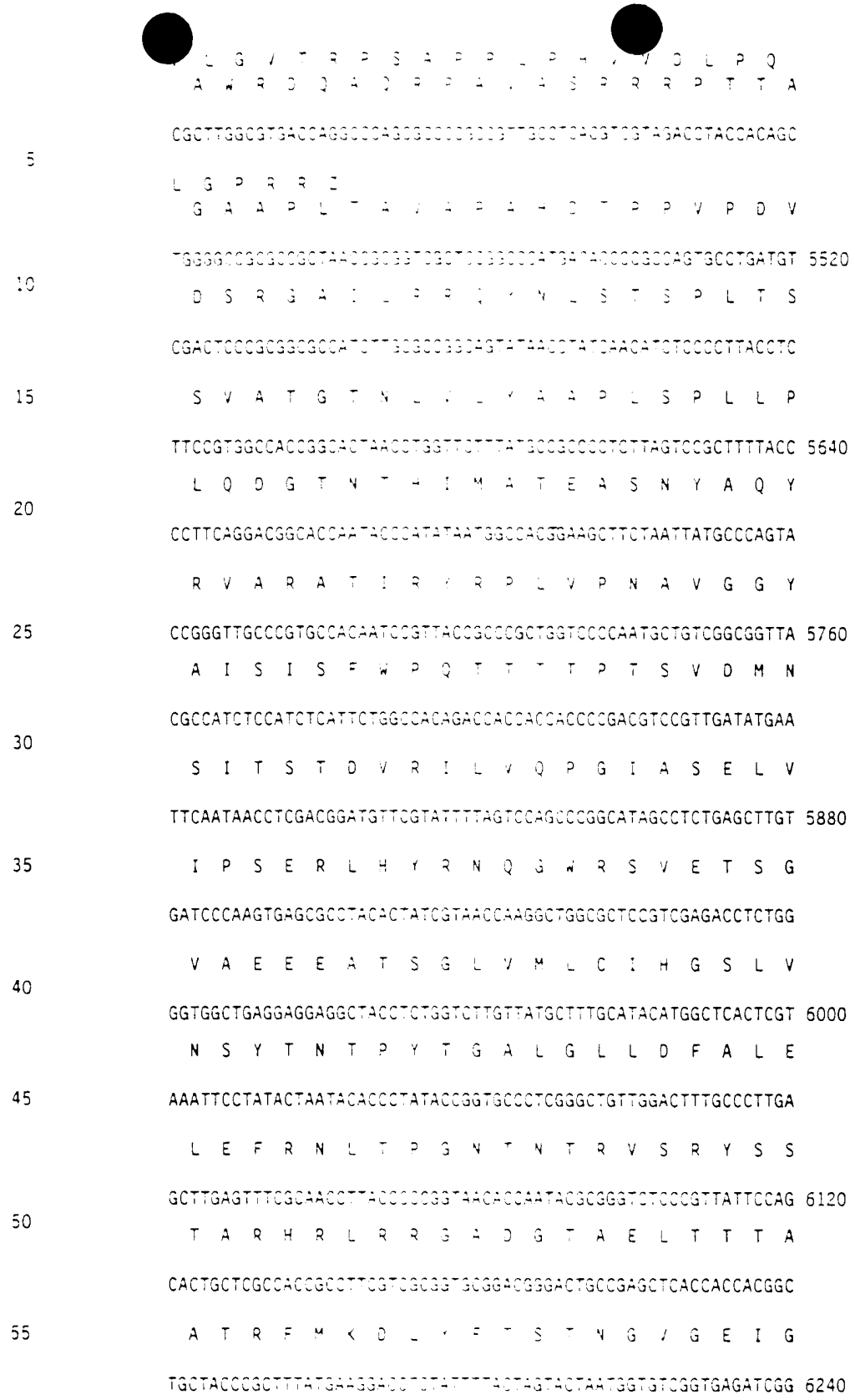

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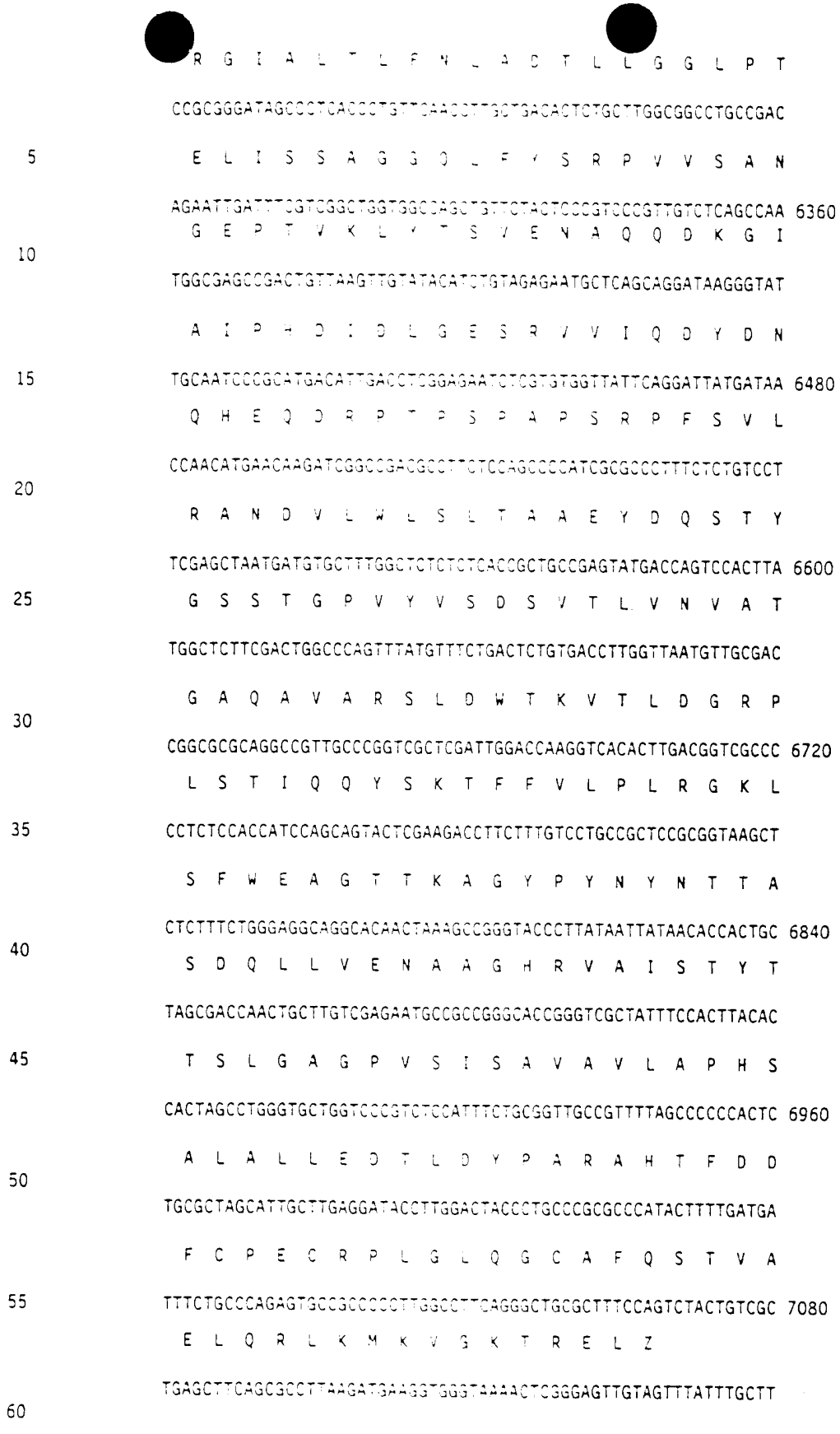
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Total number of bases in this sequence as presented is 7195. The poly-A tail present in the cloned sequence has been omitted.

The ability of the methods described herein to isolate and identify genetic material from other NANB hepatitis strains has been confirmed by identifying genetic material from an isolate obtained in Mexico. The sequence of this isolate was about 75% identical to the ET1.1 sequence set forth in SEQ ID NO.1 above. The sequence was identified by hybridization using the conditions set forth in Section II.B below.

In this different approach to isolation of the virus, cDNA libraries were made directly from a semi-purified human stool specimen collected from an outbreak of ET-NANB in Telixtac. The recovery of cDNA and the construction of representative libraries was assured by the application of sequence independent single premier amplification (SISPA). A cDNA library constructed in lambda gt11 from such an amplified cDNA population was screened with a serum considered to have "high" titer anti-HEV antibodies as assayed by direct immunofluorescence on liver sections from infected cynos. Two cDNA clones, denoted 406.3-2 and 406.4-2, were identified by this approach from a total of 60,000 screened. The sequence of these clones was subsequently localized to the 3' half of the viral genome by homology comparison to the HEV (Burma) sequence obtained from clones isolated by hybridization screening of libraries with the original ET1.1 clone.

These isolated cDNA epitopes when used as hybridization probes on Northern blots of RNA extracted from infected cyno liver gave a somewhat different result when compared to the Northern blots obtained with the ET1.1 probe. In addition to the single 7.5 Kb transcript seen using ET1.1, two

additional transcripts of 3.7 and 2.0 Kb were identified using either of these epitopes as hybridization probes. These polyadenylated transcripts were identified using the extreme 3' end epitope clone (406.3-2) as probe and therefore established these transcripts as co-terminal with the 3' end of the genome (see below). One of the epitope clones (406.4-2) was subsequently shown to react in a specific fashion with antisera collected from 5 different geographic epidemics (Somalia, Burma, Mexico, Tashkent and Pakistan). The 406.3-2 clone reacted with sera from 4 out of these same 5 epidemics (Yarbough et al., 1990). Both clones reacted with only post inoculation antisera from infected cynos. The latter experiment confirmed that seroconversion in experimentally infected cynos was related to the isolated exogenous cloned sequence.

A composite cDNA sequence (obtained from several clones of the Mexican strain) is set forth below.

Composite Mexico strain sequence (SEQ ID NO.10):

SEQ ID NO. 10:

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CCTGGCACCT ACCGGACATC ATCCTACTTG CTGATCCACG ATGGTAAGCG CGCGGTTGTC	660
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GGCTGTCACT TTGTGTTGTT GATCACTGCG GCCCCTGAGC CCTCCCCGAT GCCCTACGTT 840

CCTTACCCGC GTTCGACGGA GGTCTATGTC CGGTCTATCT TTGGGCCCGG CGGGTCCCCG 900

TCGCTGTTCC CGACCGCTTG TGGTGTCAAG TCCACTTTTC ACGCCGTCCC CACGCACATC 960

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CCCTGCCCTG AGCTTGAGCA GGGCCTTCTC TATCTGCCAC AGGAGCTAGC CTCCTGTGAC 3840

AGTGTTGTGA CATTTGAGCT AACTGACATT GTGCACTGCC GCATGGCGGC CCCTAGCCAA 3900

AGGAAAGCTG TTTTGTCCAC GCTGGTAGGC CGGTATGGCA GACGCACAAG GCTTTATGAT 3960

GCGGGTCACA CCGATGTCCG CGCCTCCCTT GCGCGCTTTA TTCCCACTCT CGGGCGGGTT 4020

ACTGCCACCA CCTGTGAACT CTTTGAGCTT GTAGAGGCGA TGGTGGAGAA GGGCCAAGAC 4080

GGTTCAGCCG TCCTCGAGTT GGATTTGTGC AGCCGAGATG TCTCCCGCAT AACCTTTTTC 4140

CAGAAGGATT GTAACAAGTT CACGACCGGC GAGACAATTG CGCATGGCAA AGTCGGTCAG 4200

GGTATCTTCC GCTGGAGTAA GACGTTTTGT GCCCTGTTTG GCCCCTGGTT CCGTGCGATT 4260

GAGAAGGCTA TTCTATCCGT TTTACCACAA GCTGTGTTCT ACGGGGATGC TTATGACGAC 4320

TCAGTATTCT CTGCTGCCGT GGCTGGCGCC AGCCATGCCA TGGTGTTTGA AAATGATTTT 4380

TCTGAGTTTG ACTCGACTCA GAATAACTTT TCCCTAGGTC TTGAGTGCGC CATTATGGAA 4440

GAGTGTGGTA TGCCCCAGTG GCTTGTCAGG TTGTACCATG CCGTCCGGTC GGCGTGGATC 4500

CTGCAGGCCC CAAAAGAGTC TTTGAGAGGG TTCTGGAAGA AGCATTCTGG TGAGCCGGGC 4560

AGCTTGCTCT GGAATACGGT GTGGAACATG GCAATCATTG CCCATTGCTA TGAGTTCCGG 4620

GACCTCCAGG TTGCCGCCTT CAAGGGCGAC GACTCGGTCG TCCTCTGTAG TGAATACCGC 4680

CAGAGCCCAG GCGCCGGTTC GCTTATAGCA GGCTGTGGTT TGAAGTTGAA GGCTGACTTC 4740

CGGCCGATTG GGCTGTATGC CGGGGTTGTC GTCGCCCCGG GGCTCGGGGC CCTACCCGAT 4800

GTCGTTCGAT TCGCCGGACG GCTTTCGGAG AAGAACTGGG GGCCTGATCC GGAGCGGGCA 4860

GAGCAGCTCC GCCTCGCCGT GCAGGATTTC CTCCGTAGGT TAACGAATGT GGCCCAGATT 4920

TGTGTTGAGG TGGTGTCTAG AGTTTACGGG GTTTCCCCGG GTCTGGTTCA TAACCTGATA 4980

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CGGTACCGGC GGTGGTTTCT GGGGTGACCG GGTTGATTCT CAGCCCTTCG CAATCCCCTA 5280

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CGCTGCCTCC CGTCGCCGAC CTGCCACAGC CGGGGCTGCG GCGCTGACGG CTGTGGCGCC 5460

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TGTTCAACCT GGCATAGCAT CTGAATTGGT CATCCCAAGC GAGCGCCTTC ACTACCGCAA 5880

TCAAGGTTGG CGCTCGGTTG AGACATCTGG TGTTGCTGAG GAGGAAGCCA CCTCCGGTCT 5940

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CACCAATACA CGTGTGTCCC GTTACTCCAG CACTGCTCGT CACTCCGCCC GAGGGGCCGA 6120

CGGGACTGCG GAGCTGACCA CAACTGCAGC CACCAGGTTC ATGAAAGATC TCCACTTTAC 6180

CGGCCTTAAT GGGGTAGGTG AAGTCGGCCG CGGGATAGCT CTAACATTAC TTAACCTTGC 6240

TGACACGCTC CTCGGCGGGC TCCCGACAGA ATTAATTTCG TCGGCTGGCG GGCAACTGTT 6300

TTATTCCCGC CCGGTTGTCT CAGCCAATGG CGAGCCAACC GTGAAGCTCT ATACATCAGT 6360

GGAGAATGCT CAGCAGGATA AGGGTGTTGC TATCCCCCAC GATATCGATC TTGGTGATTC 6420

GCGTGTGGTC ATTCAGGATT ATGACAACCA GCATGAGCAG GATCGGCCCA CCCCGTCGCC 6480

TGCGCCATCT CGGCCTTTTT CTGTTCTCCG AGCAAATGAT GTACTTTGGC TGTCCCTCAC 6540

TGCAGCCGAG TATGACCAGT CCACTTACGG GTCGTCAACT GGCCCGGTTT ATATCTCGGA 6600

CAGCGTGACT TTGGTGAATG TTGCGACTGG CGCGCAGGCC GTAGCCCGAT CGCTTGACTG 6660

GTCCAAAGTC ACCCTCGACG GGCGGCCCCT CCCGACTGTT GAGCAATATT CCAAGACATT 6720

CTTTGTGCTC CCCCTTCGTG GCAAGCTCTC CTTTTGGGAG GCCGGCACAA CAAAAGCAGG 6780

TTATCCTTAT AATTATAATA CTACTGCTAG TGACCAGATT CTGATTGAAA ATGCTGCCGG 6840

CCATCGGGTC GCCATTTCAA CCTATACCAC CAGGCTTGGG GCCGGTCCGG TCGCCATTTC 6900

TGCGGCCGCG GTTTTGGCTC CACGCTCCGC CCTGGCTCTG CTGGAGGATA CTTTTGATTA 6960

TCCGGGGCGG GCGCACACAT TTGATGACTT CTGCCCTGAA TGCCGCGCTT TAGGCCTCCA 7020

GGGTTGTGCT TTCCAGTCAA CTGTCGCTGA GCTCCAGCGC CTTAAAGTTA AGGTGGGTAA 7080

AACTCGGGAG TTGTAGTTTA TTTGGCTGTG CCCACCTACT TATATCTGCT GATTTCCTTT 7140

ATTTCCTTTT TCTCGGTCCC GCGCTCCCTG A 7171

The above sequence was obtained from polyadenylated clones. For clarity the 3' polyA "tail" has been omitted.

The sequence above includes a partial cDNA sequence consisting of 1661 nucleotides that was identified in a previous application in this series. The previously identified partial sequence is set forth below, with certain corrections (SEQ ID NO.11). The corrections include deletion of the first 80 bases of the prior reported sequence, which are cloning artifacts; insertion of G after former position 174, of C after 270, and of GGCG after 279; change of C to T at former position 709, of GC to CG at 722-723, of CC to TT at 1238-39, and of C to G at 1606; deletion of T at former position 765; and deletion of the last 11 bases of the former sequence, which are part of a linker sequence and are not of viral origin.

Non-A Non-B T: Mexican Strain; SEQ ID NO.11

SEQ ID NO. 11:

GTTGCGTGAG GTGGGTATCT CAGATGCCAT TGTTAATAAT TTCTTCCTTT CGGGTGGCGA 60

GGTTGGTCAC CAGAGACCAT CGGTCATTCC GCGAGGCAAC CCTGACCGCA ATGTTGACGT 120

GCTTGCGGCG TTTCCACCTT CATGCCAAAT AAGCGCCTTC CATCAGCTTG CTGAGGAGCT 180

GGGCCACCGG CCGGCGCCGG TGGCGGCTGT GCTACCTCCC TGCCCTGAGC TTGAGCAGGG 240

CCTTCTCTAT CTGCCACAGG AGCTAGCCTC CTGTGACAGT GTTGTGACAT TTGAGCTAAC 300

TGACATTGTG CACTGCCGCA TGGCGGCCCC TAGCCAAAGG AAAGCTGTTT TGTCCACGCT 360

GGTAGGCCGG TATGGCAGAC GCACAAGGCT TTATGATGCG GGTCACACCG ATGTCCGCGC 420

CTCCCTTGCG CGCTTTATTC CCACTCTCGG GCGGGTTACT GCCACCACCT GTGAACTCTT 480

TGAGCTTGTA GAGGCGATGG TGGAGAAGGG CCAAGACGGT TCAGCCGTCC TCGAGTTGGA 540

TTTGTGCAGC CGAGATGTCT CCCGCATAAC CTTTTTCCAG AAGGATTGTA ACAAGTTCAC 600

GACCGGCGAG ACAATTGCGC ATGGCAAAGT CGGTCAGGGT ATCTTCCGCT GGAGTAAGAC 660

CTTTTGTGCC CTGTTTGGCC CCTGGTTCGG TGCGATTGAG AAGGCTATTC TATCCCTTTT 720

ACCACAAGCT GTGTTCTACG GGGATGCTTA TGACGACTCA GTATTCTCTG CTGCCGTGGC 780

TGGCGCCAGC CATGCCATGG TGTTTGAAAA TGATTTTTCT GAGTTTGACT CGACTCAGAA 840

TAACTTTTCC CTAGGTCTTG AGTGCGCCAT TATGGAAGAG TGTGGTATGC CCCAGTGGCT 900

TGTCAGGTTG TACCATGCCG TCGGGTCGGC GTGGATCCTG CAGGCCCCAA AAGAGTCTTT 960

GAGAGGGTTC TGGAAGAAGC ATTCTGGTGA GCCGGGCACG TTGCTCTGGA ATACGGTGTG 1020

GAACATGGCA ATGATTGCCC ATTGCTATGA GTTCCGGGAC CTCCAGGTTG CCGCCTTCAA 1080

GGGCGACGAC TCGGTCGTCC TCTGTAGTGA ATACCGCCAG AGCCCAGGCG CCGGTTCGCT 1140

TATAGCAGGC TGTGGTTTGA AGTTGAAGGC TGACTTCCGG CCGATTGGGC TGTATGCCGG 1200

GGTTGTCGTC GCCCCGGGGC TCGGGGCCCT ACCCGATGTC GTTCGATTCG CCGGACGGCT 1260

TTCGGAGAAG AACTGGGGGC CTGATCCGGA GCGGGCAGAG CAGCTCCGCC TCGCCGTGCA 1320

GGATTTCCTC CGTAGGTTAA CGAATGTGGC CCAGATTTGT GTTGAGGTGG TGTCTAGAGT 1380

TTACGGGGTT TCCCCGGGTC TGGTTCATAA CCTGATAGGC ATGCTCCAGA CTATTGGTGA 1440

TGGTAAGGCG CATTTTACAG AGTCTGTTAA GCCTATACTT GACCTTACAC ACTCAATTAT 1500

GCACCGGTCT GAATGAATAA CATGTGGTTT GCTGCGCCCA TGGGTTCGCC ACCATGCGCC 1560

CTAGGCCTCT TTTGC 1575

When comparing the Burmese and Mexican strains, 75.7% identity is seen in a 7189 nucleotide overlap beginning at nucleotide 1 of the Mexican strain and nucleotide 25 of the Burmese strain.

In the same manner, a different strain of HEV was identified in an isolate obtained in Tashkent, U.S.S.R. The Tashkent sequence is given below (SEQ ID NO.12):

SEQ ID NO. 12:

CGGGCCCCGT ACAGGTCACA ACCTGTGAGT TGTACGAGCT AGTGGAGGCC ATGGTCGAGA 60

AAGGCCAGGA TGGCTCCGCC GTCCTTGAGC TCGATCTCTG CAACCGTGAC GTGTCCAGGA 120

TCACCTTTTT CCAGAAAGAT TGCAATAAGT TCACCACGGG AGAGACCATC GCCCATGGTA 180

AAGTGGGCCA GGGCATTTCG GCCTGGAGTA AGACCTTCTG TGCCCTTTTC GGCCCCTGGT 240

TCCGTGCTAT TGAGAAGGCT ATTCTGGCCC TGCTCCCTCA GGGTGTGTTT TATGGGGATG 300

CCTTTGATGA CACCGTCTTC TCGGCGCGTG TGGCCGCAGC AAAGGCGTCC ATGGTGTTTG 360

AGAATGACTT TTCTGAGTTT GACTCCACCC AGAATAATTT TTCCCTGGGC CTAGAGTGTG 420

CTATTATGGA GAAGTGTGGG ATGCCGAAGT GGCTCATCCG CTTGTACCAC CTTATAAGGT 480

CTGCGTGGAT CCTGCAGGCC CCGAAGGAGT CCCTGCGAGG GTGTTGGAAG AAACACTCCG 540

GTGAGCCCGG CACTCTTCTA TGGAATACTG TGTGGAACAT GGCCGTTATC ACCCATTGTT 600

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ACGATTTCCG CGATTTGCAG GTGGCTGCCT TTAAAGGTGA TGATTCGATA GTGCTTTGCA   660

GTGAGTACCG TCAGAGTCCA GGGGCTGCTG TCCTGATTGC TGGCTGTGGC TTAAAGCTGA   720

AGGTGGGTTT CCGTCCGATT GGTTTGTATG CAGGTGTTGT GGTGACCCCC GGCCTTGGCG   780

CGCTTCCCGA CGTCGTGCGC TTGTCCGGCC GGCTTACTGA GAAGAATTGG GGCCCTGGCC   840

CTGAGCGGGC GGAGCAGCTC CGCCTTGCTG TGCG                               874
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As shown in the following comparison of sequences, the Tashkent (Tash.) sequence more closely resembles the Burma sequence than the Mexico sequence, as would be expected of two strains from more closely related geographical areas. The numbering system used in the comparison is based on the Burma sequence. As indicated previously, Burma has SEQ ID NO:6; Mexico, SEQ ID NO:10; and Tashkent, SEQ ID NO:12. The letters present in the lines between the sequences indicate conserved nucleotides.

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                  10v        20v        30v        40v        50v        60v
-BURMA    AGGCAGACCACATATGTGGTCGATGCCATGGAGGCCCATCAGTTTATTAAGGCTCCTGGCA
                                     GCCATGGAGGCCCA CAGTT ATTAAGGCTCCTGGCA
-MEXICO                              GCCATGGAGGCCCACCAGTTCATTAAGGCTCCTGGCA

                  70v        80v        90v       100v       110v       120v
-BURMA    TCACTACTGCTATTGAGCAGGCTGCTCTAGCAGCGGCCAACTCTGCCCTGGCGAATGCTG
                  TCACTACTGCTATTGAGCA GC GCTCTAGCAGCGGCCAACTC GCCCT GCGAATGCTG
-MEXICO   TCACTACTGCTATTGAGCAAGCAGCTCTAGCAGCGGCCAACTCCGCCCTTGCGAATGCTG

                 130v       140v       150v       160v       170v       180v
-BURMA    TGGTAGTTAGGCCTTTTCTCTCTCACCAGCAGATTGAGATCCTCATTAACCTAATGCAAC
                  TGGT GT  GGCCTTT CT TC CA CAGCAG TTGAGATCCT AT AA CT ATGCAAC
-MEXICO   TGGTGGTCCGGCCTTTCCTTTCCCATCAGCAGGTTGAGATCCTTATAAATCTCATGCAAC

                 190v       200v       210v       220v       230v       240v
-BURMA    CTCGCCAGCTTGTTTTCCGCCCCGAGGTTTTCTGGAATCATCCCATCCAGCGTGTCATCC
                  CTCG CAGCT GT TT CG CC GAGGTTTT TGGAATCA CC AT CA CGTGT AT C
-MEXICO   CTCGGCAGCTGGTGTTTCGTCCTGAGGTTTTTTGGAATCACCCGATTCAACGTGTTATAC

                 250v       260v       270v       280v       290v       300v
-BURMA    ATAACGAGCTGGAGCTTTACTGCCGCGCCCGCTCCGGCCGCTGTCTTGAAATTGGCGCCC
                  ATAA GAGCT GAGC  TA TGCCG GC CGCTC GG CGCTG CTTGA ATTGG GCCC
-MEXICO   ATAATGAGCTTGAGCAGTATTGCCGTGCTCGCTCGGGTCGCTGCCTTGAGATTGGAGCCC

                 310v       320v       330v       340v       350v       360v
-BURMA    ATCCCCGCTCAATAAATGATAATCCTAATGTGGTCCACCGCTGCTTCCTCCGCCCTGTTG
                  A CC CGCTC AT AATGATAATCCTAATGT  TCCA CGCTGCTT CTCC CCC GT G
-MEXICO   ACCCACGCTCCATTAATGATAATCCTAATGTCCTCCATCGCTGCTTTCTCCACCCCGTCG
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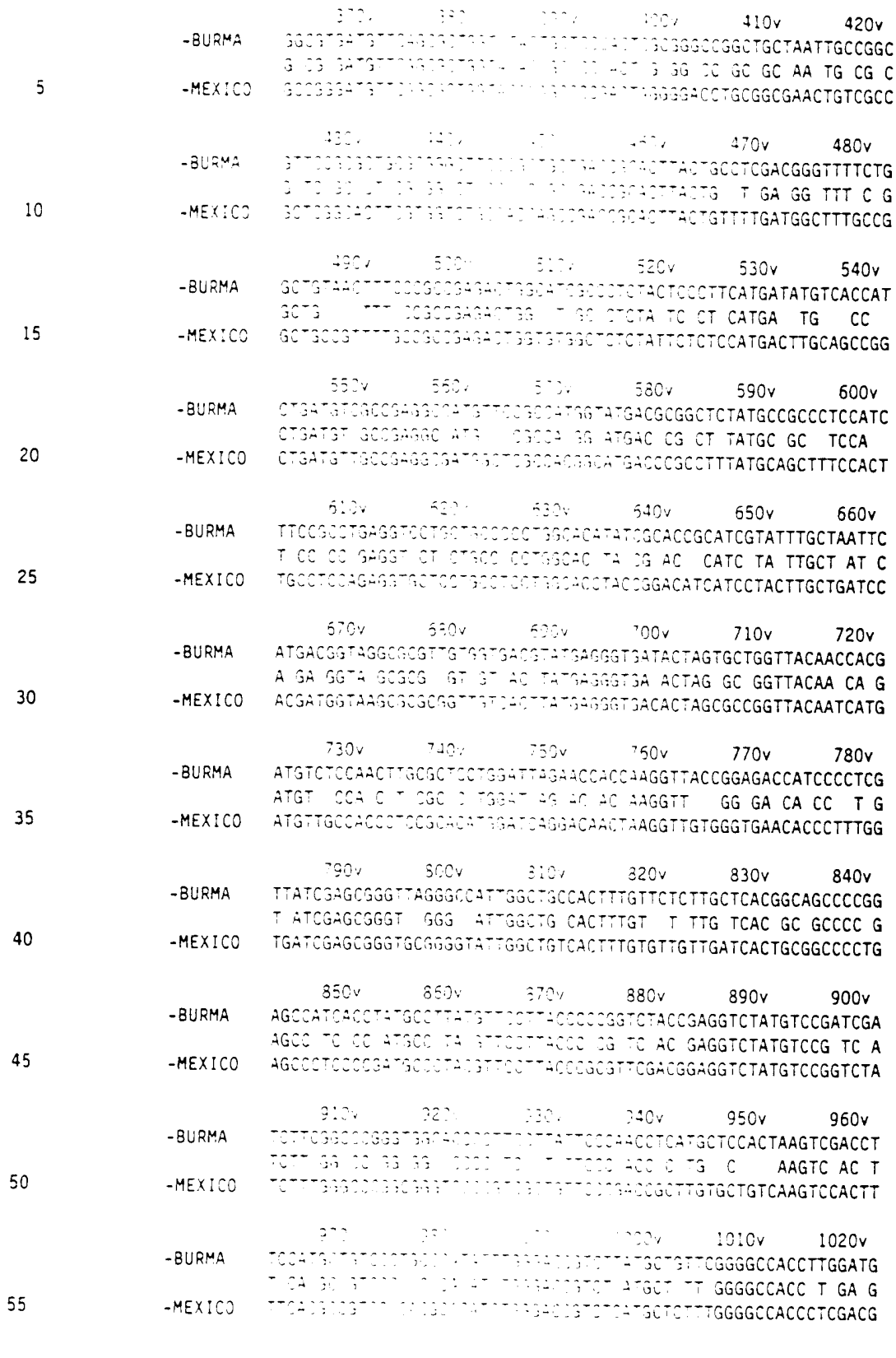
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-MEXICO
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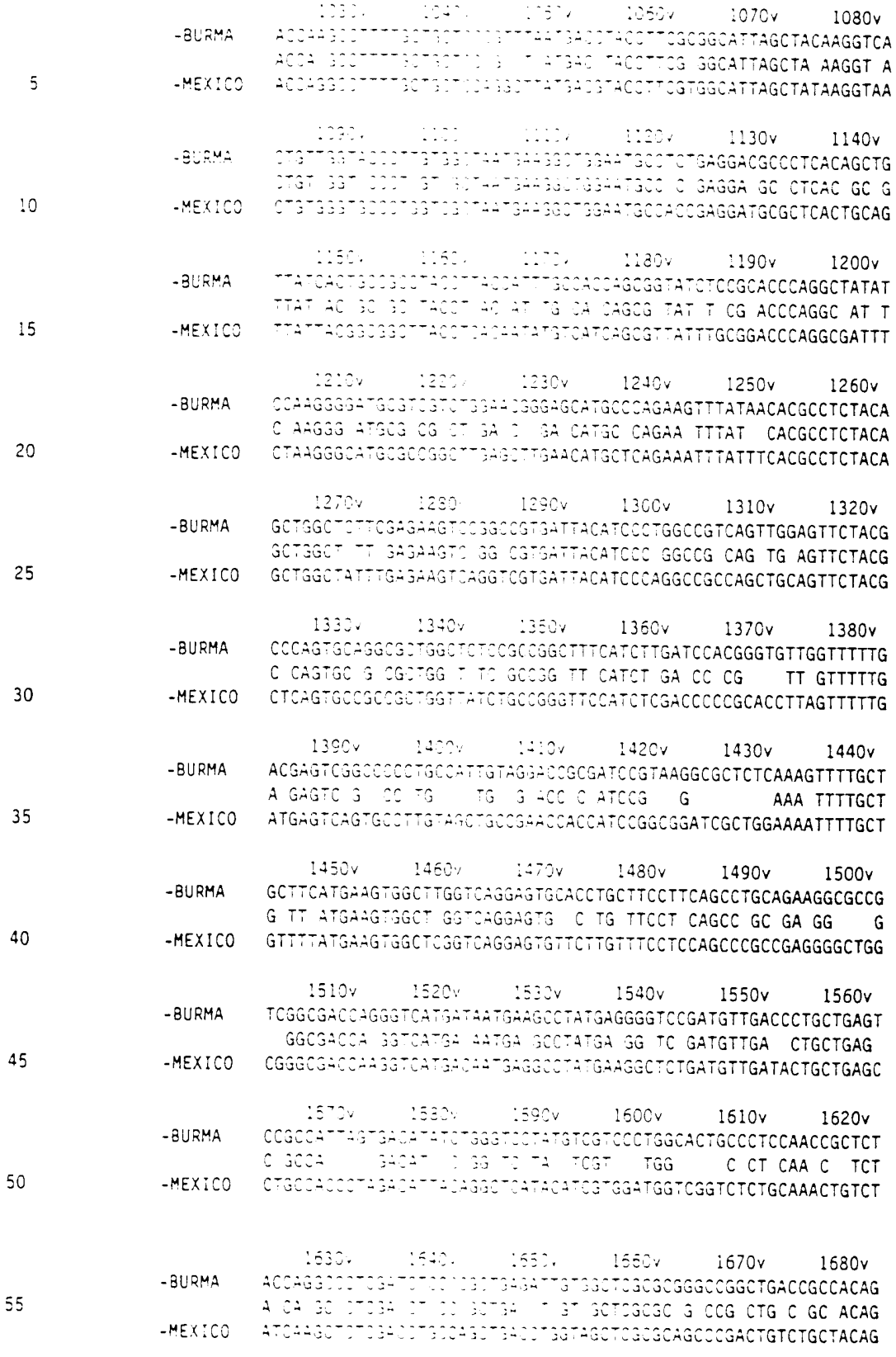
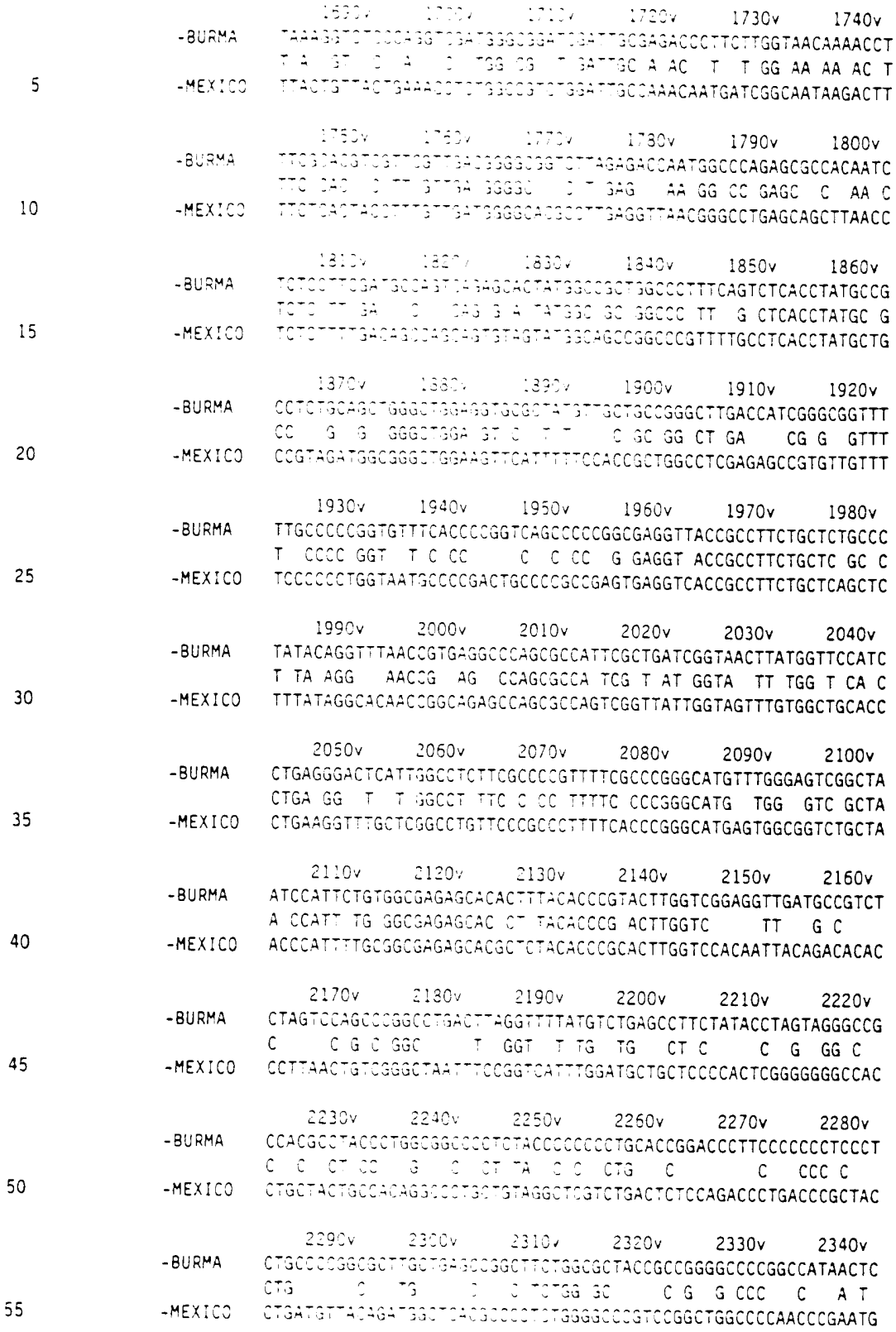
-MEXICO
490v 500v 510v 520v 530v 540v
-BURMA
-MEXICO
550v 560v 570v 580v 590v 600v
-BURMA
-MEXICO
610v 620v 630v 640v 650v 660v
-BURMA
-MEXICO
670v 680v 690v 700v 710v 720v
-BURMA
-MEXICO
730v 740v 750v 760v 770v 780v
-BURMA
-MEXICO
790v 800v 810v 820v 830v 840v
-BURMA
-MEXICO
850v 860v 870v 880v 890v 900v
-BURMA
-MEXICO
910v 920v 930v 940v 950v 960v
-BURMA
-MEXICO
970v 980v 990v 1000v 1010v 1020v
-BURMA
-MEXICO

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-BURMA TAAAGGTCTCCCAGGTCGATGGCGGATCGATTGCGAGACCCTTCTTGGTAACAAAACCT
T A GT C A C TGG CG T GATTGC A AC T T GG AA AA AC T
5 -MEXICO TTACTGTTACTGAAACCTCTGGCCGTCTGGATTGCCAAACAATGATCGGCAATAAGACTT
1750v 1760v 1770v 1780v 1790v 1800v
-BURMA TTCGCACGTCGTTCGTTGACGGGGCGGTCTTAGAGACCAATGGCCCAGAGCGCCACAATC
TTC GAG C TT GTTGA GGGGC C T GAG AA GG CC GAGC C AA C
10 -MEXICO TTCTCAGTACGTTTGTTGATGGGGCACGCCTTGAGGTTAACGGGCCTGAGCAGCTTAACC
1810v 1820v 1830v 1840v 1850v 1860v
-BURMA TCTCCTTCGATGCCATTCATGAGCACTATGGCCGCTGGCCCTTTCAGTCTCACCTATGCCG
TCTC TT GA C GAG G A TATGGC GC GGCCC TT G CTCACCTATGC G
15 -MEXICO TCTCTTTTGACAGCCAGCAGTGTAGTATGGCAGCCGGCCCGTTTTGCCTCACCTATGCTG
1870v 1880v 1890v 1900v 1910v 1920v
-BURMA CCTCTGCAGCTGGGCTGGAGGTGCGCTATGTTGCTGCCGGGCTTGACCATCGGGCGGTTT
CC G G GGGCTGGA GT C T T C GC GG CT GA CG G GTTT
20 -MEXICO CCGTAGATGGCGGGCTGGAAGTTCATTTTTCCACCGCTGGCCTCGAGAGCCGTGTTGTTT
1930v 1940v 1950v 1960v 1970v 1980v
-BURMA TTGCCCCCGGTGTTTCACCCCGGTCAGCCCCCGGCGAGGTTACCGCCTTCTGCTCTGCCC
T CCCC GGT T C CC C C CC G GAGGT ACCGCCTTCTGCTC GC C
25 -MEXICO TCCCCCCTGGTAATGCCCCGACTGCCCCGCCGAGTGAGGTCACCGCCTTCTGCTCAGCTC
1990v 2000v 2010v 2020v 2030v 2040v
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T TA AGG AACCG AG CCAGCGCCA TCG T AT GGTA TT TGG T CA C
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CTGA GG T T GGCCT TTC C CC TTTTC CCCGGGCATG TGG GTC GCTA
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2110v 2120v 2130v 2140v 2150v 2160v
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40 -MEXICO ACCCATTTTGCGGCGAGAGCACGCTCTACACCCGCACTTGGTCCACAATTACAGACACAC
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C C G C GGC T GGT T TG TG CT C C G GG C
45 -MEXICO CCTTAACTGTCGGGCTAATTTCCGGTCATTTGGATGCTGCTCCCCACTCGGGGGGGCCAC
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C C CT CC G C CT TA C C CTG C C CCC C
50 -MEXICO CTGCTACTGCCACAGGCCCTGCTGTAGGCTCGTCTGACTCTCCAGACCCTGACCCGCTAC
2290v 2300v 2310v 2320v 2330v 2340v
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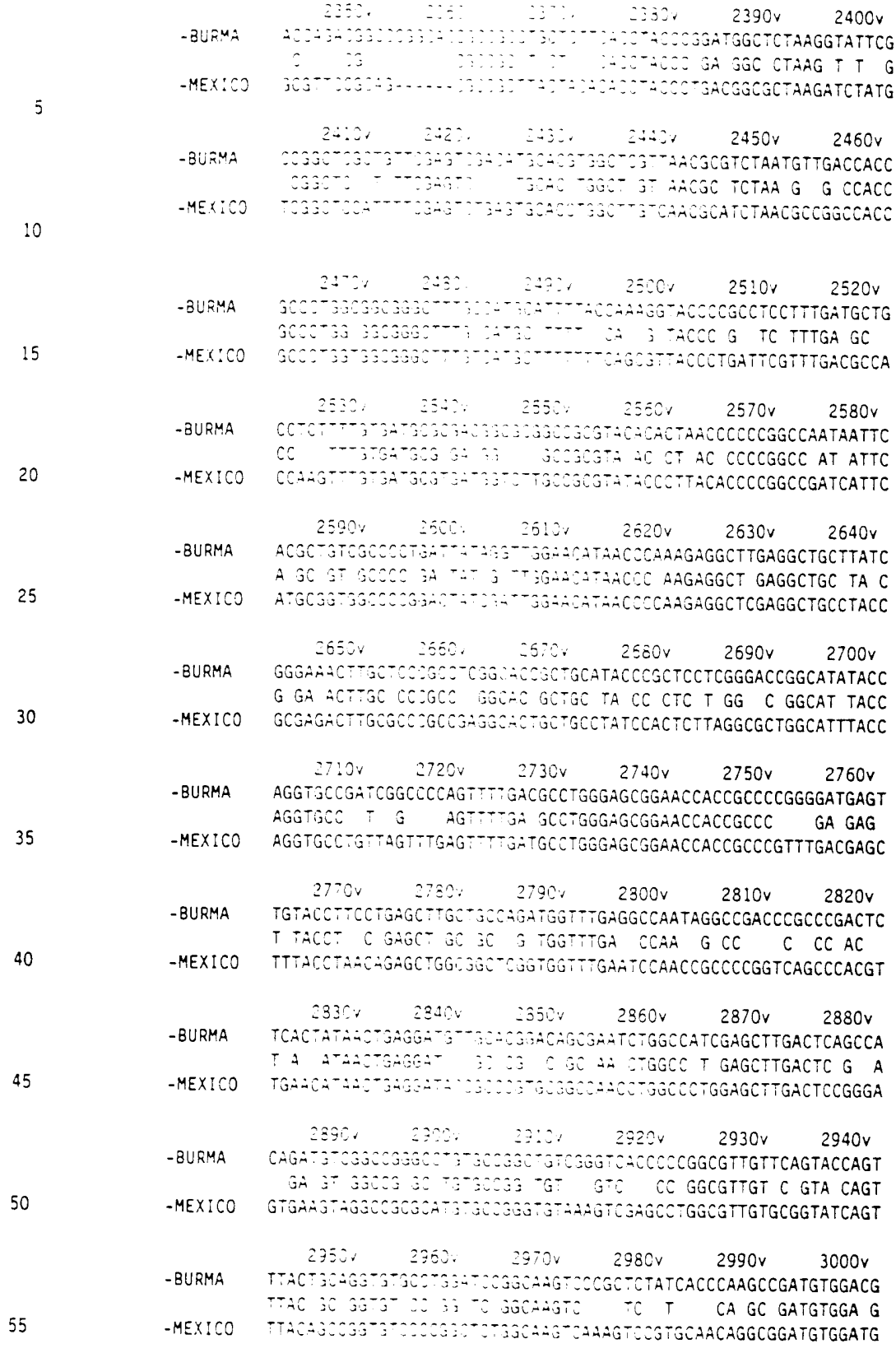
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                    3010v     3020v     3030v     3040v     3050v     3060v
-BURMA      TTGTCGTGGTCCCGACGCGTGAGTTGCGTAATGCCTGGCGCCGTCGCGGCTTTGCTGCTT
            TTGT GT GT CC AC CG GAG T CG AA GC TGGCG CG CG GGCTTTGC GC T
-MEXICO     TTGTTGTTGTGCCCACTCGCGAGCTTCGGAACGCTTGGCGGCGCCGGGGCTTTGCGGCAT

                    3070v     3080v     3090v     3100v     3110v     3120v
-BURMA      TTACCCCGCATACTGTCCCCAGAGTCACCCAGGGGCGCCGGGTTGTCATTGATGAGGCTC
            T AC CCGCA ACTGC CCC G GTCAC    GG CG  GGGTTGTCATTGATGAGGC C
-MEXICO     TCACTCCGCACACTGTCCCCCGTGTCACTAGCGGCGTAGGGTTGTCATTGATGAGGCCC

                    3130v     3140v     3150v     3160v     3170v     3180v
-BURMA      CATCCCTCCCCCCTCACCTGCTGCTGCTCCACATGCAGCGGGCCGCCACCGTCCACCTTC
            C TC CTCCCCCC CAC TGCTGC  T CA ATGCAGCG GC GC  C GT CACCT C
-MEXICO     CTTCGCTCCCCCCACACTTGCTGCTTTTACATATGCAGCGTGCTGCATCTGTGCACCTCC

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-MEXICO     TTGGGGACCCGAATCAGATCCCCGCCATAGATTTTGAGCACACCGGTCTGATTCCAGCAA

                    3250v     3260v     3270v     3280v     3290v     3300v
-BURMA      TCAGGCCCGACTTAGGCCCCACCTCCTGGTGGCATGTTACCCATCGCTGGCCTGCGGATG
            T  GGCC GA TT G CCC AC TC TGGTGGCATGT ACCCA CG TG CCTGC GATG
-MEXICO     TACGGCCGGAGTTGGTCCCGACTTCATGGTGGCATGTCACCCACCGTTGCCCTGCAGATG

                    3310v     3320v     3330v     3340v     3350v     3360v
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            T TG GAG T  TCCGTGGTGC TACCC A  ATCCAGAC AC AG   GGT CTCCGTT
-MEXICO     TCTGTGAGTTAGTCCGTGGTGCTTACCCTAAAATCCAGACTACAAGTAAGGTGCTCCGTT

                    3370v     3380v     3390v     3400v     3410v     3420v
-BURMA      CGTTGTTCTGGGGTGAGCCTGCCGTCGGGCAGAAACTAGTGTTCACCCAGGCGGCCAAGC
            C  T TTCTGGGG GAGCC GC GTCGG CAGAA CTAGTGTTCAC CAGGC GC AAG
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-MEXICO     CCGCGCACCCCGGATCTATAACGGTCCATGAGGCCCAGGGTGCCACTTTTACCACTACAA

                    3490v     3500v     3510v     3520v     3530v     3540v
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            CTAT ATTGC AC GCAGATGCCCG GGCCT AT CAGTC TC CGGGCTCA GC AT G
-MEXICO     CTATAATTGCAACTGCAGATGCCCGTGGCCTCATACAGTCCTCCCGGGCTCACGCTATAG

                    3550v     3560v     3570v     3580v     3590v     3600v
-BURMA      TTGCTCTGACGCGCCACACTGAGAAGTGCGTCATCATTGACGCACCAGGCCTGCTTCGCG
            TTGCTCT AC  G CA ACTGA AA TG GT AT  TTGAC C CC GGCCTG T CG G
-MEXICO     TTGCTCTCACTAGGCATACTGAAAAATGTGTTATACTTGACTCTCCCGGCCTGTTGCGTG

                    3610v     3620v     3630v     3640v     3650v     3660v
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            AGGTGGG ATCTC GATTC AT GTTAATAA TT TTCCT  C GGTGGCGA  TTGGTC
-MEXICO     AGGTGGGTATCTCAGATTCCATTGTTAATAATTTCTTCCTTTCGGGTGGCGAGGTTGGTC
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           C TT CC CC TC TGCCA AT AG GCCTTCCATCAG T GCTGAGGAGCT GGCCAC
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           G CC G  CC GT GC GCTGT CTACC CCCTGCCC GAGCT GA CAGGGCCTTCTCT
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                    3850v     3860v     3870v     3880v     3890v     3900v
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                    3910v     3920v     3930v     3940v     3950v     3960v
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-MEXICO    GGTATGGCAGACGCACAAGGCTTTATGATGCGGGTCACACCGATGTCCGCGCCTCCCTTG

                    4030v     4040v     4050v     4060v     4070v     4080v
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           C CG TTTAT CC  C  T GG C  GT    G  AC AC TGTGAA T T  GAGCT G
-MEXICO    CGCGCTTTATTCCCACTCTCGGGCGGGTTACTGCCACCACCTGTGAACTCTTTGAGCTTG

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-BURMA     TGGAGGCCATGGTCGAGAAGGGCCAGGATGGCTCCGCCGTCCTTGAGCTTGATCTTTGCA
           T GAGGC ATGGT GAGAAGGGCCA GA GG TC GCCGTCCT GAG T GAT T TGCA
-MEXICO    TAGAGGCGATGGTGGAGAAGGGCCAAGACGGTTCAGCCGTCCTCGAGTTGGATTTGTGCA

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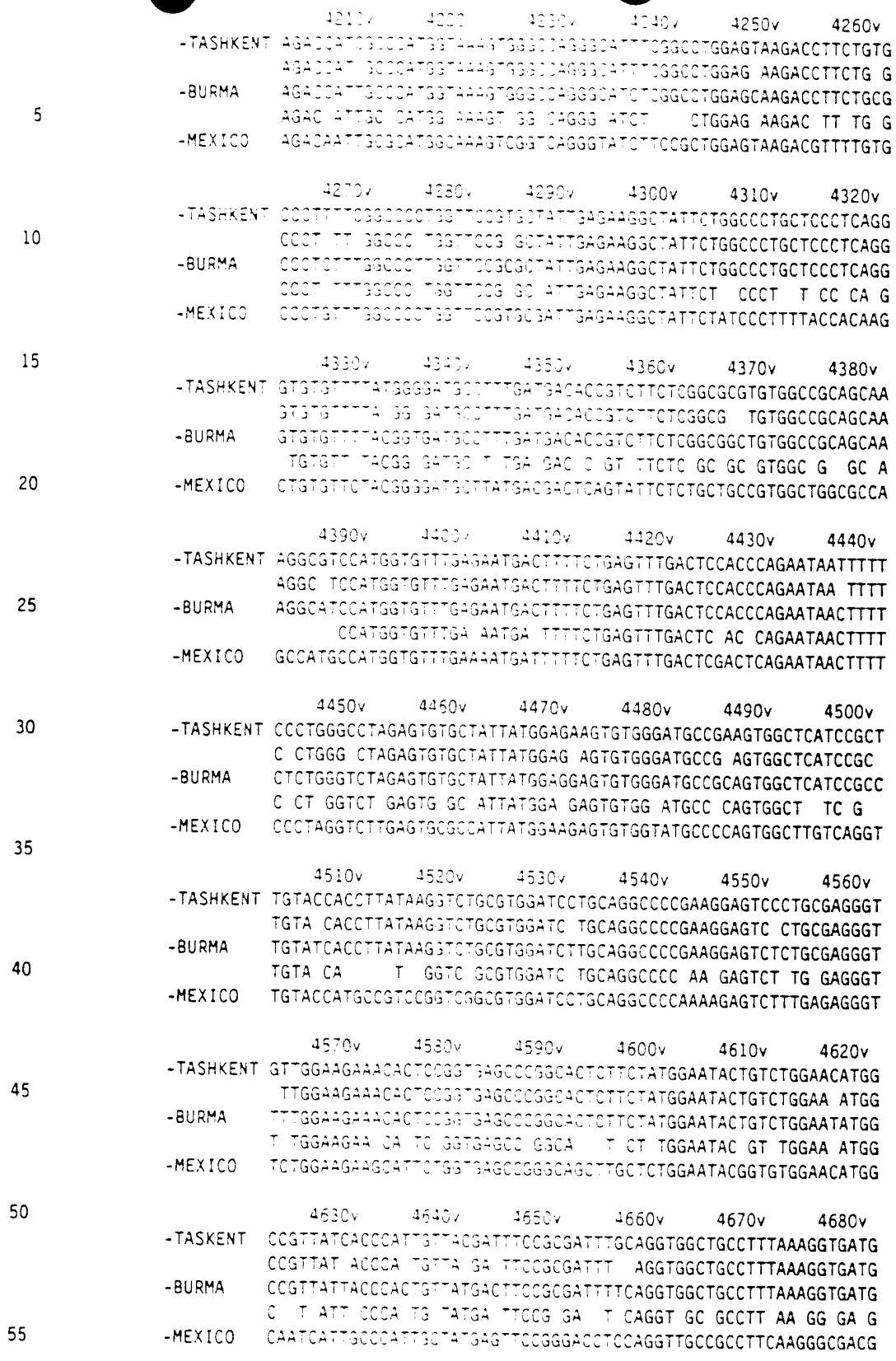
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-MEXICO GCCATGCCATGGTGTTTGAAAATGATTTTTCTGAGTTTGACTCGACTCAGAATAACTTTT
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C CT GGTCT GAGTG GC ATTATGGA GAGTGTGG ATGCC CAGTGGCT TC G
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          GCTGTGGCTT AAG TGAAGT G TTCCG CCGAT GGTTTGTATGCAGGTGTTGTGG
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          GCTGTGG TTGAAGTTGAAGG GA TTCCG CCGAT GG  TGTATGC GG GTTGT G
-MEXICO   GCTGTGGTTTGAAGTTGAAGGCTGACTTCCGGCCGATTGGGCTGTATGCCGGGGTTGTCG

                    4810v      4820v      4830v      4840v      4850v      4860v
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          TG CCCCCGGCCTTGGCGCGCTTCCCGA GT GTGCGCTTG CCGGCCGGCTTAC GAGA
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-MEXICO   TCGCCCCCGGGGCTCGGGGCCCTACCCGATGTCGTTCGATTCGCCGGACGGCTTTCGGAGA

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          TCCG A G T ACGAATGT GC CAGAT TGTGT GA GT GT TC  G GTTTA GGGG
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          C CATTT AC GAGTC GT AA CC  T CT GAC T ACA A TCAAT  TG   CGG
-MEXICO   CGCATTTTACAGAGTCTGTTAAGCCTATACTTGACCTTACACACTCAATTATGCACCGGT

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            GAATGAATAACATGT  TTTGCTGCGCCCATGGGTTCGC ACCATGCGCCCT GGCCT
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                    5230v     5240v     5250v     5260v     5270v     5280v
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           TCTGGCCGCCGTCGTGGGCGGCGCAGCGGCGGT CCGGCGGTGGTTTCTGGGGTGACCGG
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           GT  CCGCTGCG CCGGG TTGGACCTCG  TTCGCCAACC GCCCG CCACT GGCTCC
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           TC GTGGCC C GGCACTAA  T GT CT TATGC GCCCC CTTA TCCGC T T CC
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                    5650v     5660v     5670v     5680v     5690v     5700v
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                    5710v     5720v     5730v     5740v     5750v     5760v
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                    5770v     5780v     5790v     5800v     5810v     5820v
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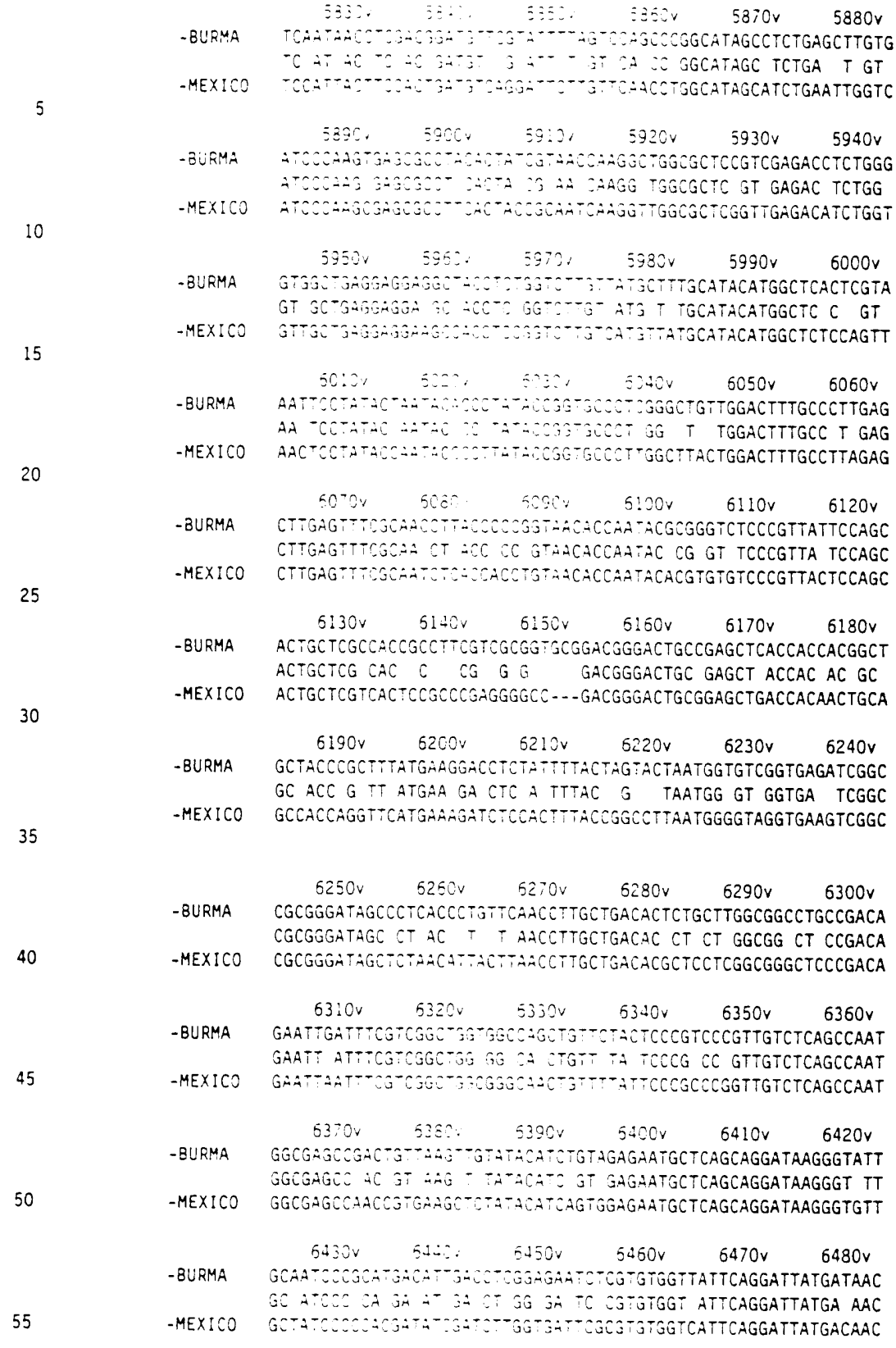
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TC AT AC TC AC GATGT G ATT T GT CA CC GGCATAGC TCTGA T GT
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AA TCCTATAC AATAC CC TATACCGGTGCCCT GG T TGGACTTTGCC T GAG
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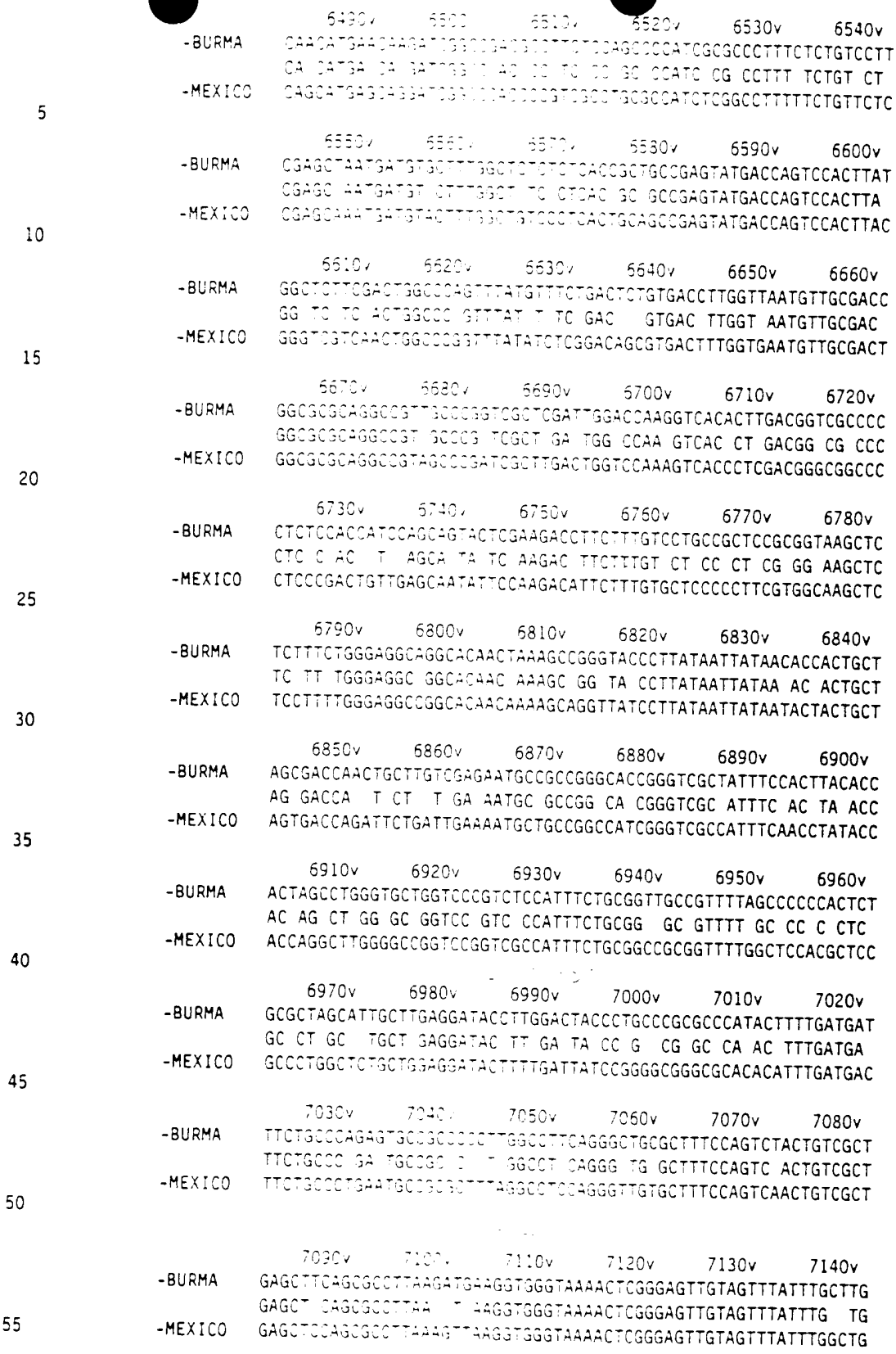
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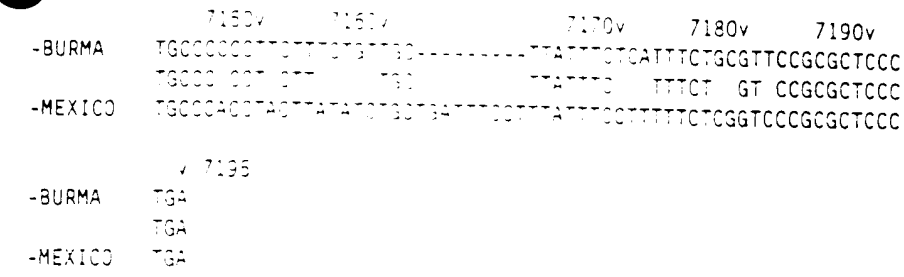

A number of open reading frames, which are potential coding regions, have been found within the DNA sequences set forth above. As has already been noted, consensus residues for the RNA-directed RNA polymerase (RDRP) were identified in the HEV (Burma) strain clone ET1.1. Once a contiguous overlapping set of clones was accumulated, it became clear that the nonstructural elements containing the RDRP as well as what were identified as consensus residues for the helicase domain were located in the first large open reading frame (ORFI). ORFI covers the 5' half of the genome and begins at the first encoded met, after the 27th bp of the apparent non-coding sequence, and then extends 5079 bp before reaching a termination codon. Beginning 37 bp downstream from the ORF1 stop codon in the plus 1 frame is the second major opening reading frame (ORF2) extending 1980 bp and terminating 68 bp upstream from the point of poly A addition. The third forward ORF (in the plus 2 frame) is also utilized by HEV. ORF3 is only 370 bp in length and would not have been predicted to be utilized by the virus were it not for the identification of the immunoreactive cDNA clone 406.4-2 from the Mexico SISPA cDNA library (see below for detailed discussion). This epitope confirmed the utilization of ORF3 by the virus, although the means by which this ORF is expressed has not yet been fully elucidated. If we assume that the first met is utilized, ORF3 overlaps ORF1 by 1 bp at its 5' end and ORF2 by 328 bp at its 3'end. ORF2 contains the broadly reactive 406.3-2 epitope and also

a signal sequence at its extreme 5′ end. The first half of this ORF2 also has a high pI value (>10) similar to that seen with other virus capsid proteins. These data suggest that the ORF2 might be the predominant structural gene of HEV.

The existence of subgenomic transcripts prompted a set of experiments to determine whether these RNAs were produced by splicing from the 5′ end of the genome. An analysis using subgenomic probes from throughout the genome, including the extreme 5′ end, did not provide evidence for a spliced transcript. However, it was discovered that a region of the genome displayed a high degree of homology with a 21 bp segment identified in Sindbis as a probably internal initiation site for RNA transcription used in the production of its subgenomic messages. Sixteen of 21 (76%) of the nucleotides are identical.

Two cDNA clones which encode an epitope of HEV that is recognized by sera collected from different ET-NANB outbreaks (i.e., a universally recognized epitope) have been isolated and characterized. One of the clones immunoreacted with 8 human sera from different infected individuals and the other clone immunoreacted with 7 of the human sera tested. Both clones immunoreacted specifically with cyno sera from infected animals and exhibited no immunologic response to sera from uninfected animals. The sequences of the cDNAs in these recombinant phages, designated 406.3-2 and 406.4-2 have been determined. The HEV open reading frames are shown to encode epitopes specifically recognized by sera from patients with HEV infections. The cDNA sequences and the polypeptides that they encode are set forth below.

Epitopes derived from Mexican strain of HEV:

406.4-2 sequence (nucleotide sequence has SEQ ID NO.13; amino acid sequence has SEQ ID NO.14):

SEQ ID NO. 13:

```
C GCC AAC CAG CCC GGC CAC TTG GCT CCA CTT GGC GAG ATC AGG CCC          46
  Ala Asn Gln Pro Gly His Leu Ala Pro Leu Gly Glu Ile Arg Pro
    1               5                  10                  15

AGC GCC CCT CCG CTG CCT CCC GTC GCC GAC CTG CCA CAG CCG GGG CTG        94
Ser Ala Pro Pro Leu Pro Pro Val Ala Asp Leu Pro Gln Pro Gly Leu
                20                  25                  30

CGG CGC TGA CGGCTGTGGC GCCTGCCCAT GACACCTCAC CCGTCCCGGA               143
Arg Arg  .

CGTTGATTCT CGCGGTGCAA TTCTACGCCG CCAGTATAAT TTGTCTACTT CACCCCTGAC     203

ATCCTCTGTG GCCTCTGGCA CTAATTTAGT CCTGTATGCA GCCCCCCTTA ATCCGCCTCT     263

GCCGCTGCAG GACGGTACTA ATACTCACAT TATGGCCACA GAGGCCTCCA ATTATGCACA     323

GTACCGGGTT GCCCGCGCTA CTATCCGTTA CCGGCCCCTA GTGCCTAATG CAGTTGGAGG     383

CTATGCTATA TCCATTTCTT TCTGGCCTCA AACAACCACA ACCCCTACAT CTGTTGACAT     443

GAATTC                                                               449
```

SEQ ID NO. 14:

```
Ala Asn Gln Pro Gly His Leu Ala Pro Leu Gly Glu Ile Arg Pro Ser
  1               5                  10                  15

Ala Pro Pro Leu Pro Pro Val Ala Asp Leu Pro Gln Pro Gly Leu Arg
            20                  25                  30

Arg  .
```

406.3-2 sequence (nucleotide sequence has SEQ ID NO.15; amino acid sequence has SEQ ID NO.16):

SEQ ID NO. 15:

```
GGAT ACT TTT GAT TAT CCG GGG CGG GCG CAC ACA TTT GAT GAC TTC TGC      49
     Thr Phe Asp Tyr Pro Gly Arg Ala His Thr Phe Asp Asp Phe Cys
       1               5                  10                  15

CCT GAA TGC CGC GCT TTA GGC CTC CAG GGT TGT GCT TTC CAG TCA ACT       97
Pro Glu Cys Arg Ala Leu Gly Leu Gln Gly Cys Ala Phe Gln Ser Thr
                20                  25                  30

GTC GCT GAG CTC CAG CGC CTT AAA GTT AAG GTT                          130
Val Ala Glu Leu Gln Arg Leu Lys Val Lys Val
            35                  40
```

SEQ ID NO. 16:

Thr Phe Asp Tyr Pro Gly Arg Ala His Thr Phe Asp Asp Phe Cys Pro
1 5 10 15

Glu Cys Arg Ala Leu Gly Leu Gln Gly Cys Ala Phe Gln Ser Thr Val
20 25 30

Ala Glu Leu Gln Arg Leu Lys Val Lys Val
35 40

The universal nature of these epitopes is evident from the homology exhibited by the DNA that encodes them. If the epitope coding sequences from the Mexican strains shown above are compared to DNA sequences from other strains, such as the Burmese strain also set forth above, similarities are evident, as shown in the following comparisons.

Comparison of 406.4-2 epitopes, HEV Mexico and Burma strains:

```
                                    10        20        30
MEXICAN(SEQ ID NO.17)      ANQPGHLAPLGEIRPSAPPLPPVADLPQPGLRR
                           ::.:.: :::: .:::::::::.:.:::: : ::
BURMA(SEQ ID NO.18)        ANPPDHSAPLGVTRPSAPPLPHVVDLPQLGPRR
                                    10        20        30
```

There is 73.5% identity in a 33-amino acid overlap.

Comparison of 406.3-2 epitopes, HEV Mexico and Burma strains:

MEXICAN(SEQ ID No.19)

```
                    10        20        30        40
              TFDYPGRAHTFDDFCPECRALGLQGCAFQSTVAELQRLKVKV
              :.::::.:::::::::::::.:::::::::::::::::.::
              TLDYPARAHTFDDFCPECRPLGLQGCAFQSTVAELQRLKMKV
                    10        20        30        40
```

BURMA(SEQ ID No.20)

There is 90.5% identity in the 42-amino acid overlap.

It will be recognized by one skilled in the art of molecular genetics that each of the specific DNA sequences given above shows a corresponding complementary DNA sequence as well as RNA sequences corresponding to both the principal sequence shown and

the complementary DNA sequence. Additionally, open reading frames encoding peptides are present, and expressible peptides are disclosed by the nucleotide sequences without setting forth the amino acid sequences explicitly, in the same manner as if the amino acid sequences were explicitly set forth as in the ET1.1 sequence or other sequences above.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

The terms defined below have the following meaning herein:

1. "Enterically transmitted non-A/non-B hepatitis viral agent, ET-NANB, or HEV" means a virus, virus type, or virus class which (i) causes water-borne, infectious hepatitis, (ii) is transmissible in cynomolgus monkeys, (iii) is serologically distinct from hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus, and (iv) includes a genomic region which is homologous to the 1.33 kb cDNA insert in plasmid pTZKF1(ET1.1) carried in *E. coli* strain BB4 identified by ATCC deposit number 67717.

2. Two nucleic acid fragments are "homologous" if they are capable of hybridizing to one another under hybridization conditions described in Maniatis *et al.*, *op. cit.*, pp. 320-323. However, using the following wash conditions: 2 x SCC, 0.1% SDS, room temperature twice, 30 minutes each; then 2 x SCC, 0.1% SDS, 50°C once, 30 minutes; then 2 x SCC, room temperature twice, 10 minutes each, homologous sequences can be identified that contain at most about 25-30% basepair mismatches. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches. These degrees of homology can be selected by using more stringent wash conditions for identification of clones from gene libraries (or

other sources of genetic material), as is well known in the art.

3. Two amino acid sequences or two nucleotide sequences (in an alternative definition for homology between two nucleotide sequences) are considered homologous (as this term is preferably used in this specification) if they have an alignment score of >5 (in standard deviation units) using the program ALIGN with the mutation gap matrix and a gap penalty of 6 or greater. See Dayhoff, M.O., in Atlas of Protein Sequence and Structure (1972) Vol. 5, National Biomedical Research Foundation, pp. 101-110, and Supplement 2 to this volume, pp. 1-10. The two sequences (or parts thereof, preferably at least 30 amino acids in length) are more preferably homologous if their amino acids are greater than or equal to 50% identical when optimally aligned using the ALIGN program mentioned above.

4. A DNA fragment is "derived from" an ET-NANB viral agent if it has the same or substantially the same basepair sequence as a region of the viral agent genome.

5. A protein is "derived from" an ET-NANB viral agent if it is encoded by an open reading frame of a DNA or RNA fragment derived from an ET-NANB viral agent.

II. Obtaining Cloned ET-NANB Fragments

According to one aspect of the invention, it has been found that a virus-specific DNA clone can be produced by (a) isolating RNA from the bile of a cynomolgus monkey having a known ET-NANB infection, (b) cloning the cDNA fragments to form a fragment library, and (c) screening the library by differential hybridization to radiolabeled cDNAs from infected and non-infected bile sources.

A. cDNA Fragment Mixture

ET-NANB infection in cynomolgus monkeys is initiated by inoculating the animals intravenously with a 10% w/v suspension from human case stools positive for 27-34 nm ET-NANB particles (mean diameter 32 nm). An infected animal is monitored for elevated levels of alanine aminotransferase, indicating hepatitis infection. ET-NANB infection is confirmed by immunospecific binding of seropositive antibodies to virus-like particles (VLPs), according to published methods (Gravelle). Briefly, a stool (or bile) specimen taken from the infected animal 3-4 weeks after infection is diluted 1:10 with phosphate-buffered saline, and the 10% suspension is clarified by low-speed centrifugation and filtration successively through 1.2 and 0.45 micron filters. The material may be further purified by pelleting through a 30% sucrose cushion (Bradley). The resulting preparation of VLPs is mixed with diluted serum from human patients with known ET-NANB infection. After incubation overnight, the mixture is centrifuged overnight to pellet immune aggregates, and these are stained and examined by electron microscopy for antibody binding to the VLPs.

ET-NANB infection can also be confirmed by seroconversion to VLP-positive serum. Here the serum of the infected animal is mixed as above with 27-34 nm VLPs isolated from the stool specimens of infected human cases and examined by immune electron microscopy for antibody binding to the VLPs.

Bile can be collected from ET-NANB positive animals by either cannulating the bile duct and collecting the bile fluid or by draining the bile duct during necropsy. Total RNA is extracted from the bile by hot phenol extraction, as outlined in Example 1A. The RNA fragments are used to synthesize corresponding duplex cDNA fragments by random priming, also as referenced in Example 1A. The cDNA fragments may be fractionated by gel electrophoresis or density

gradient centrifugation to obtain a desired size class of fragments, e.g., 500-4,000 basepair fragments.

Although alternative sources of viral material, such as VLPs obtained from stool samples (as described in Example 4), may be used for producing a CDNA fraction, the bile source is preferred. According to one aspect of the invention, it has been found that bile from ET-NANB-infected monkeys shows a greater number of intact viral particles than material obtained from stool samples, as evidenced by immune electron microscopy. Bile obtained from an ET-NANB infected human or cynomolgus macaque, for use as a source of ET-NANB viral protein or genomic material, or intact virus, forms part of the present invention.

B. cDNA Library and Screening

The cDNA fragments from above are cloned into a suitable cloning vector to form a cDNA library. This may be done by equipping blunt-ended fragments with a suitable end linker, such as an EcoRI sequence, and inserting the fragments into a suitable insertion site of a cloning vector, such as at a unique EcoRI site. After initial cloning, the library may be re-cloned, if desired, to increase the percentage of vectors containing a fragment insert. The library construction described in Example 1B is illustrative. Here cDNA fragments were blunt-ended, equipped with EcoRI ends, and inserted into the EcoRI site of the lambda phage vector gt10. The library phage, which showed less than 5% fragment inserts, was isolated, and the fragment inserts re-cloned into the lambda gt10 vector, yielding more than 95% insert-containing phage.

The cDNA library is screened for sequences specific for ET-NANB by differential hybridization to cDNA probes derived from infected and non-infected sources. cDNA fragments from infected and non-infected source bile or stool viral isolates can be prepared as above. Radiolabeling the fragments is by random

labeling, nick translation, or end labeling, according to conventional methods (Maniatis, p. 109). The cDNA library from above is screened by transfer to duplicate nitrocellulose filters, and hybridization with both infected-source and non-infected-source (control) radiolabeled probes, as detailed in Example 2. In order to recover sequences that hybridize at the preferred outer limit of 25-30% basepair mismatches, clones can be selected if they hybridize under the conditions described in Maniatis et al., op. cit., pp. 320-323, but using the following wash conditions: 2 x SCC, 0.1% SDS, room temperature - twice, 30 minutes each; then 2 x SCC, 0.1% SDS, 50°C - once, 30 minutes; then 2 x SCC, room temperature - twice, 10 minutes each. These conditions allowed identification of the Mexican isolate discussed above using the ET1.1 sequence as a probe. Plaques which show selective hybridization to the infected-source probes are preferably re-plated at low plating density and re-screened as above, to isolate single clones which are specific for ET-NANB sequences. As indicated in Example 2, sixteen clones which hybridized specifically with infected-source probes were identified by these procedures. One of the clones, designated lambda gt101.1, contained a 1.33 kilobase fragment insert.

C. ET-NANB Sequences

The basepair sequence of cloned regions of the ET-NANB fragments from Part B are determined by standard sequencing methods. In one illustrative method, described in Example 3, the fragment insert from the selected cloning vector is excised, isolated by gel electrophoresis, and inserted into a cloning vector whose basepair sequence on either side of the insertion site is known. The particular vector employed in Example 3 is a pTZKF1 vector shown at the left in Figure 1. The ET-NANB fragment from the gt10-

1.1 phage was inserted at the unique EcoRI site of the pTZKF1 plasmid. Recombinants carrying the desired insert were identified by hybridization with the isolated 1.33 kilobase fragment, as described in Example 3. One selected plasmid, identified as pTZKF1 (ET1.1), gave the expected 1.33 kb fragment after vector digestion with EcoRI. E. coli strain BB4 infected with the pTZKF1(ET1.1) plasmid has been deposited with the American Type Culture Collection, Rockville, MD, and is identified by ATCC deposit number 67717.

The pTZKF1(ET1.1) plasmid is illustrated at the bottom in Figure 1. The fragment insert has 5' and 3' end regions denoted at A and C, respectively, and an intermediate region, denoted at B. The sequences in these regions were determined by standard dideoxy sequencing and were set forth in an earlier application in this series. The three short sequences (A, B, and C) are from the same insert strand. As will be seen in Example 3, the B-region sequence was actually determined from the opposite strand, so that the B region sequence shown above represents the complement of the sequence in the sequenced strand. The base numbers of the partial sequences are approximate.

Later work in the laboratory of the inventors identified the full sequence, set forth above. Fragments of this total sequence can readily be prepared using restriction endonucleases. Computer analysis of both the forward and reverse sequence has identified a number of cleavage sites.

III. ET-NANB Fragments

According to another aspect, the invention includes ET-NANB-specific fragments or probes which hybridize with ET-NANB genomic sequences or cDNA fragments derived therefrom. The fragments may include full-length cDNA fragments such as described in

Section II, or may be derived from shorter sequence regions within cloned cDNA fragments. Shorter fragments can be prepared by enzymatic digestion of full-length fragments under conditions which yield desired-sized fragments, as will be described in Section IV. Alternatively, the fragments can be produced by oligonucleotide synthetic methods, using sequences derived from the cDNA fragments. Methods or commercial services for producing selected-sequence oligonucleotide fragments are available. Fragments are usually at least 12 nucleotides in length, preferably at least 14, 20, 30 or 50 nucleotides, when used as probes. Probes can be full length or less than 500, preferably less than 300 or 200, nucleotides in length.

To confirm that a given ET-NANB fragment is in fact derived from the ET-NANB viral agent, the fragment can be shown to hybridize selectively with cDNA from infected sources. By way of illustration, to confirm that the 1.33 kb fragment in the pTZKF1(ET1.1) plasmid is ET-NANB in origin, the fragment was excised from the pTZKF1(ET1.1) plasmid, purified, and radiolabeled by random labeling. The radiolabeled fragment was hybridized with fractionated cDNAs from infected and non-infected sources to confirm that the probe reacts only with infected-source cDNAs. This method is illustrated in Example 4, where the above radiolabeled 1.33 kb fragment from pTZKF1(ET1.1) plasmid was examined for binding to cDNAs prepared from infected and non-infected sources. The infected sources are (1) bile from a cynomolgus macaque infected with a strain of virus derived from stool samples from human patients from Burma with known ET-NANB infections and (2) a viral agent derived from the stool sample of a human ET-NANB patient from Mexico. The cDNAs in each fragment mixture were first amplified by a linker primer amplification method described in Example 4. Fragment separation was on

agarose gel, followed by Southern blotting and then hybridization to bind the radiolabeled 1.33 kb fragment to the fractionated cDNAs. The lane containing cDNAs from the infected sources showed a smeared band of bound probe, as expected (cDNAs amplified by the linker/primer amplification method would be expected to have a broad range of sizes). No probe binding to the amplified cDNAs from the non-infected sources was observed. The results indicate that the 1.33 kb probe is specific for cDNA fragments associated with ET-NANB infection. This same type of study, using ET 1.1 as the probe, has demonstrated hybridization to ET-NANB samples collected from Tashkent, Somalia, Borneo and Pakistan. Secondly, the fact that the probe is specific for ET-NANB related sequences derived from different continents (Asia, Africa and North America) indicates the cloned ET-NANB Burma sequence (ET1.1) is derived from a common ET-NANB virus or virus class responsible for ET-NANB hepatitis infection worldwide.

In a related confirmatory study, probe binding to fractionated genomic fragments prepared from human or cynomolgus macaque genomic DNA (both infected and uninfected) was examined. No probe binding was observed to either genomic fraction, demonstrating that the ET-NANB fragment is not an endogenous human or cynomolgus genomic fragment and additionally demonstrating that HEV is an RNA virus.

Another confirmation of ET-NANB specific sequences in the fragments is the ability to express ET-NANB proteins from coding regions in the fragments and to demonstrated specific sero-reactivity of these proteins with sera collected during documented outbreaks of ET-NANB. Section IV below discusses methods of protein expression using the fragments.

One important use of the ET-NANB-specific fragments is for identifying ET-NANB-derived cDNAs which contain additional sequence information. The

newly identified cDNAs, in turn, yield new fragment probes, allowing further iterations until the entire viral genome is identified and sequenced. Procedures for identifying additional ET-NANB library clones and generating new probes therefrom generally follow the cloning and selection procedures described in Section II.

The fragments (and oligonucleotides prepared based on the sequences given above) are also useful as primers for a polymerase chain reaction method of detecting ET-NANB viral genomic material in a patient sample. This diagnostic method will be described in Section V below.

Two specific genetic sequences derived from the Mexican strain, identified herein as 406.3-2 and 406.4-2, have been identified that encode immunogenic epitopes. This was done by isolating clones which encode epitopes that immunologically react specifically with sera from individuals and experimental animals infected with HEV. Comparison of the isolated sequences with those in the Genebank collection of genetic sequences indicate that these viral sequences are novel. Since these sequences are unique, they can be used to identify the presence of HEV and to distinguish this strain of hepatitis from HAV, HBV, and HCV strains. The sequences are also useful for the design of oligonucleotide probes to diagnose the presence of virus in samples. They can be used for the synthesis of polypeptides that themselves are used in immunoassays. The specific 406.3-2 and 406.4-2 sequences can be incorporated into other genetic material, such as vectors, for ease of expression or replication. They can also be used (as demonstrated above) for identifying similar antigenic regions encoded by related viral strains, such as the Burmese strain.

IV. ET-NANB Proteins

As indicated above, ET-NANB proteins can be prepared by expressing open reading-frame coding regions in ET-NANB fragments. In one preferred approach, the ET-NANB fragments used for protein expression are derived from cloned cDNAs which have been treated to produce desired-size fragments, and preferably random fragments with sizes predominantly between about 100 to about 300 base pairs. Example 5 describes the preparation of such fragments by DNAs digestion. Because it is desired to obtain peptide antigens of between about 30 to about 100 amino acids, the digest fragments are preferably size fractionated, for example by gel electrophoresis, to select those in the approximately 100-300 basepair size range. Alternatively, cDNA libraries constructed directly from HEV-containing sources (e.g., bile or stool) can be screened directly if cloned into an appropriate expression vector (see below).

For example, the ET-NANB proteins expressed by the 406.3-2 and 406.4-2 sequences (and peptide fragments thereof) are particularly preferred since these proteins have been demonstrated to be immunoreactive with a variety of different human sera, thereby indicating the presence of one or more epitopes specific for HEV on their surfaces. These clones were identified by direct screening of a gt11 library.

A. Expression Vector

The ET-NANB fragments are inserted into a suitable expression vector. One exemplary expression vector is lambda gt11, which contains a unique EcoRI insertion site 53 base pairs upstream of the translation termination codon of the beta-galactosidase gene. Thus, the inserted sequence will be expressed as a beta-galactosidase fusion protein which contains the N-terminal portion of the beta-galactosidase gene, the heterologous peptide, and

optionally the C-terminal region of the beta-galactosidase peptide (the C-terminal portion being expressed when the heterologous peptide coding sequence does not contain a translation termination codon). This vector also produces a temperature-sensitive repressor (c1857) which causes viral lysogeny at permissive temperatures, e.g., 32°C, and leads to viral lysis at elevated temperatures, e.g., 37°C. Advantages of this vector include: (1) highly efficient recombinant generation, (2) ability to select lysogenized host cells on the basis of host-cell growth at permissive, but not non-permissive, temperatures, and (3) high levels of recombinant fusion protein production. Further, since phage containing a heterologous insert produces an inactive beta-galactosidase enzyme, phage with inserts can be readily identified by a beta-galactosidase colored-substrate reaction.

For insertion into the expression vector, the viral digest fragments may be modified, if needed, to contain selected restriction-site linkers, such as EcoRI linkers, according to conventional procedures. Example 1 illustrates methods for cloning the digest fragments into lambda gt11, which includes the steps of blunt-ending the fragments, ligating with EcoRI linkers, and introducing the fragments into EcoRI-cut lambda gt11. The resulting viral genomic library may be checked to confirm that a relatively large (representative) library has been produced. This can be done, in the case of the lambda gt11 vector, by infecting a suitable bacterial host, plating the bacteria, and examining the plaques for loss of beta-galactosidase activity. Using the procedures described in Example 1, about 50% of the plaques showed loss of enzyme activity.

B. Peptide Antigen Expression

The viral genomic library formed above is screened for production of peptide antigen (expressed as a fusion protein) which is immunoreactive with antiserum from ET-NANB seropositive individuals . In a preferred screening method, host cells infected with phage library vectors are plated, as above, and the plate is blotted with a nitrocellulose filter to transfer recombinant protein antigens produced by the cells onto the filter. The filter is then reacted with the ET-NANB antiserum, washed to remove unbound antibody, and reacted with reporter-labeled, anti-human antibody, which becomes bound to the filter, in sandwich fashion, through the anti-ET-NANB antibody.

Typically phage plaques which are identified by virtue of their production of recombinant antigen of interest are re-examined at a relatively low density for production of antibody-reactive fusion protein. Several recombinant phage clones which produced immunoreactive recombinant antigen were identified in the procedure.

The selected expression vectors may be used for scale-up production, for purposes of recombinant protein purification. Scale-up production is carried out using one of a variety of reported methods for (a) lysogenizing a suitable host, such as E. coli, with a selected lambda gt11 recombinant (b) culturing the transduced cells under conditions that yield high levels of the heterologous peptide, and (c) purifying the recombinant antigen from the lysed cells.

In one preferred method involving the above lambda gt11 cloning vector, a high-producer E. coli host, BNN103, is infected with the selected library phage and replica plated on two plates. One of the plates is grown at 32°C, at which viral lysogeny can occur, and the other at 42°C, at which the infecting phage is in a lytic stage and therefore prevents cell growth. Cells which grow at the lower but not the

higher temperature are therefore assumed to be successfully lysogenized.

The lysogenized host cells are then grown under liquid culture conditions which favor high production of the fused protein containing the viral insert, and lysed by rapid freezing to release the desired fusion protein.

C. Peptide Purification

The recombinant peptide can be purified by standard protein purification procedures which may include differential precipitation, molecular sieve chromatography, ion-exchange chromatography, isoelectric focusing, gel electrophoresis and affinity chromatography. In the case of a fused protein, such as the beta-galactosidase fused protein prepared as above, the protein isolation techniques which are used can be adapted from those used in isolation of the native protein. Thus, for isolation of a soluble betagalactosidase fusion protein, the protein can be isolated readily by simple affinity chromatography, by passing the cell lysis material over a solid support having surface-bound anti-beta-galactosidase antibody.

D. Viral Proteins

The ET-NANB protein of the invention may also be derived directly from the ET-NANB viral agent. VLPs or protein isolated from stool or liver samples from an infected individual, as above, are one suitable source of viral protein material. The VLPs isolated from the stool sample may be further purified by affinity chromatography prior to protein isolation (see below). The viral agent may also be raised in cell culture, which provides a convenient and potentially concentrated source of viral protein. Co-owned U.S. Patent Application Serial No. 846,757, filed April 1, 1986, describes an immortalized trioma

liver cell which supports NANB infection in cell culture. The trioma cell line is prepared by fusing human liver cells with a mouse/human fusion partner selected for human chromosome stability. Cells containing the desired NANB viral agent can be identified by immunofluorescence methods, employing anti-ET-NANB human antibodies.

The viral agent is disrupted, prior to protein isolation, by conventional methods, which can include sonication, high- or low-salt conditions, or use of detergents.

Purification of ET-NANB viral protein can be carried out by affinity chromatography, using a purified anti-ET-NANB antibody attached according to standard methods to a suitable solid support. The antibody itself may be purified by affinity chromatography, where an immunoreactive recombinant ETNANB protein, such as described above, is attached to a solid support, for isolation of anti-ET-NANB antibodies from an immune serum source. The bound antibody is released from the support by standard methods.

Alternatively, the anti-ET-NANB antibody may be an antiserum or a monoclonal antibody (Mab) prepared by immunizing a mouse or other animal with recombinant ETNANB protein. For Mab production, lymphocytes are isolated from the animal and immortalized with a suitable fusion partner, and successful fusion products which react with the recombinant protein immunogen are selected. These in turn may be used in affinity purification procedures, described above, to obtain native ET-NANB antigen.

V. Utility

Although ET-NANB is primarily of interest because of its effects on humans, recent data has shown that this virus is also capable of infecting other animals, especially mammals. Accordingly, any

discussion herein of utility applies to both human and veterinary uses, especially commercial veterinary uses, such as the diagnosis and treatment of pigs, cattle, sheep, horses, and other domesticated animals.

A. Diagnostic Methods

The particles and antigens of the invention, as well as the genetic material, can be used in diagnostic assays. Methods for detecting the presence of ET-NANB hepatitis comprise analyzing a biological sample such as a blood sample, stool sample or liver biopsy specimen for the presence of an analyte associated with ET-NANB hepatitis virus.

The analyte can be a nucleotide sequence which hybridizes with a probe comprising a sequence of at least about 16 consecutive nucleotides, usually 30 to 200 nucleotides, up to substantially the full sequence of the sequences shown above (cDNA sequences). The analyte can be RNA or cDNA. The analyte is typically a virus particle suspected of being ET-NANB or a particle for which this classification is being ruled out. The virus particle can be further characterized as having an RNA viral genome comprising a sequence at least about 70% homologous to a sequence of at least 12 consecutive nucleotides of the "forward" and "reverse" sequences given above, usually at least about 80% homologous to at least about 60 consecutive nucleotides within the sequences, and may comprise a sequence substantially homologous to the full-length sequences. In order to detect an analyte, where the analyte hybridizes to a probe, the probe may contain a detectable label. Particularly preferred for use as a probe are sequences of consecutive nucleotides derived from the 406.3-2 and 406.4-2 clones described herein, since these clones appear to be particularly diagnostic for HEV.

The analyte can also comprise an antibody which recognizes an antigen, such as a cell surface

antigen, on a ET-NANB virus particle. The analyte can also be a ET-NANB viral antigen. Where the analyte is an antibody or an antigen, either a labelled antigen or antibody, respectively, can be used to bind to the analyte to form an immunological complex, which can then be detected by means of the label.

Typically, methods for detecting analytes such as surface antigens and/or whole particles are based on immunoassays. Immunoassays can be conducted either to determine the presence of antibodies in the host that have arisen from infection by ET-NANB hepatitis virus or by assays that directly determine the presence of virus particles or antigens. Such techniques are well known and need not be described here in detail. Examples include both heterogeneous and homogeneous immunoassay techniques. Both techniques are based on the formation of an immunological complex between the virus particle or its antigen and a corresponding specific antibody. Heterogeneous assays for viral antigens typically use a specific monoclonal or polyclonal antibody bound to a solid surface. Sandwich assays are becoming increasingly popular. Homogeneous assays, which are carried out in solution without the presence of a solid phase, can also be used, for example by determining the difference in enzyme activity brought on by binding of free antibody to an enzyme-antigen conjugate. A number of suitable assays are disclosed in U.S. Patent Nos. 3,817,837, 4,006,360, 3,996,345.

When assaying for the presence of antibodies induced by ET-NANB viruses, the viruses and antigens of the invention can be used as specific binding agents to detect either IgG or IgM antibodies. Since IgM antibodies are typically the first antibodies that appear during the course of an infection, when IgG synthesis may not yet have been initiated, specifically distinguishing between IgM and IgG antibodies present in the blood stream of a host will

enable a physician or other investigator to determine whether the infection is recent or convalescent. Proteins expressed by the 406.3-2 and 406.4-2 clones described herein and peptide fragments thereof are particularly preferred for use as specific binding agents to detect antibodies since they have been demonstrated to be reactive with a number of different human HEV sera. Further, they are reactive with both acute and convalescent sera.

In one diagnostic configuration, test serum is reacted with a solid phase reagent having surface-bound ET-NANB protein antigen. After binding anti-ET-NANB antibody to the reagent and removing unbound serum components by washing, the reagent is reacted with reporter-labeled anti-human antibody to bind reporter to the reagent in proportion to the amount of bound anti-ET-NANB antibody on the solid support. The reagent is again washed to remove unbound labeled antibody, and the amount of reporter associated with the reagent is determined. Typically, the reporter is an enzyme which is detected by incubating the solid phase in the presence of a suitable fluorometric or colorimetric substrate.

The solid surface reagent in the above assay prepared by known techniques for attaching protein material to solid support material, such as polymeric beads, dip sticks, or filter material. These attachment methods generally include non-specific adsorption of the protein to the support or covalent attachment of the protein, typically through a free amine group, to a chemically reactive group on the solid support, such as an activate carboxyl, hydroxyl, or aldehyde group.

In a second diagnostic configuration, known as a homogeneous assay, antibody binding to a solid support produces some change in the reaction medium which can be directly detected in the medium. Known general types of homogeneous assays proposed

heretofore include (a) spin-labeled reporters, where antibody binding to the antigen is detected by a change in reported mobility (broadening of the spin splitting peaks), (b) fluorescent reporters, where binding is detected by a change in fluorescence efficiency, (c) enzyme reporters, where antibody binding effects enzyme/substrate interactions, and (d) liposome-bound reporters, where binding leads to liposome lysis and release of encapsulated reporter. The adaptation of these methods to the protein antigen of the present invention follows conventional methods for preparing homogeneous assay reagents.

In each of the assays described above, the assay method involves reacting the serum from a test individual with the protein antigen and examining the antigen for the presence of bound antibody. The examining may involve attaching a labeled anti-human antibody to the antibody being examined, either IgM (acute phase) or IgG (convalescent phase), and measuring the amount of reporter bound to the solid support, as in the first method, or may involve observing the effect of antibody binding on a homogeneous assay reagent, as in the second method.

Also forming part of the invention is an assay system or kit for carrying out the assay method just described. The kit generally includes a support with surface-bound recombinant protein antigen which is (a) immunoreactive with antibodies present in individuals infected with enterically transmitted nonA/nonB viral agent and (b) derived from a viral hepatitis agent whose genome contains a region which is homologous to the 1.33 kb DNA EcoRI insert present in plasmid pTZKF1(ET1.1) carried in E. Coli strain BB4, and having ATCC deposit no. 67717. A reporter-labeled anti-human antibody in the kit is used for detecting surface-bound anti-ET-NANB antibody.

B. Viral Genome Diagnostic Applications

The genetic material of the invention can itself be used in numerous assays as probes for genetic material present in naturally occurring infections. One method for amplification of target nucleic acids, for later analysis by hybridization assays, is known as the polymerase chain reaction or PCR technique. The PCR technique can be applied to detecting virus particles of the invention in suspected pathological samples using oligonucleotide primers spaced apart from each other and based on the genetic sequence set forth above. The primers are complementary to opposite strands of a double stranded DNA molecule and are typically separated by from about 50 to 450 nt or more (usually not more than 2000 nt). This method entails preparing the specific oligonucleotide primers and then repeated cycles of target DNA denaturation, primer binding, and extension with a DNA polymerase to obtain DNA fragments of the expected length based on the primer spacing. Extension products generated from one primer serve as additional target sequences for the other primer. The degree of amplification of a target sequence is controlled by the number of cycles that are performed and is theoretically calculated by the simple formula 2^n where n is the number of cycles. Given that the average efficiency per cycle ranges from about 65% to 85%, 25 cycles produce from 0.3 to 4.8 million copies of the target sequence. The PCR method is described in a number of publications, including Saiki et al., Science (1985) 230:1350-1354; Saiki et al., Nature (1986) 324:163-166; and Scharf et al., Science (1986) 233:1076-1078. Also see U.S. Patent Nos. 4,683,194; 4,683,195; and 4,683,202.

The invention includes a specific diagnostic method for determination of ET-NANB viral agent, based on selective amplification of ET-NANB fragments. This method employs a pair of single-strand primers derived

from non-homologous regions of opposite strands of a DNA duplex fragment, which in turn is derived from an enterically transmitted viral hepatitis agent whose genome contains a region which is homologous to the 1.33 kb DNA EcoRI insert present in plasmid pTZKF1(ET1.1) carried in E. coli strain BB4, and having ATCC deposit no. 67[illegible]. These "primer fragments," which form one aspect of the invention, are prepared from ET-NANB fragments such as described in Section III above. The method follows the process for amplifying selected nucleic acid sequences as disclosed in U.S. Patent No. 4,683,202, as discussed above.

C. Peptide Vaccine

Any of the antigens of the invention can be used in preparation of a vaccine. A preferred starting material for preparation of a vaccine is the particle antigen isolated from bile. The antigens are preferably initially recovered as intact particles as described above. However, it is also possible to prepare a suitable vaccine from particles isolated from other sources or non-particle recombinant antigens. When non-particle antigens are used (typically soluble antigens), proteins derived from the viral envelope or viral capsid are preferred for use in preparing vaccines. These proteins can be purified by affinity chromatography, also described above.

If the purified protein is not immunogenic per se, it can be bound to a carrier to make the protein immunogenic. Carriers include bovine serum albumin, keyhole limpet hemocyanin and the like. It is desirable, but not necessary, to purify antigens to be substantially free of human protein. However, it is more important that the antigens be free of proteins, viruses, and other substances not of human origin that may have been introduced by way of, or contamination of, the nutrient medium, cell lines,

tissues, or pathological fluids from which the virus is cultured or obtained.

Vaccination can be conducted in conventional fashion. For example, the antigen, whether a viral particle or a protein, can be used in a suitable diluent such as water, saline, buffered salines, complete or incomplete adjuvants, and the like. The immunogen is administered using standard techniques for antibody induction, such as by subcutaneous administration of physiologically compatible, sterile solutions containing inactivated or attenuated virus particles or antigens. An immune response producing amount of virus particles is typically administered per vaccinizing injection, typically in a volume of one milliliter or less.

A specific example of a vaccine composition includes, in a pharmacologically acceptable adjuvant, a recombinant protein or protein mixture derived from an enterically transmitted nonA/nonB viral hepatitis agent whose genome contains a region which is homologous to the 1.33 kb DNA EcoRI insert present in plasmid pTZKF1(ET1.1) carried in E. coli strain BB4, and having ATCC deposit no. 67717. The vaccine is administered at periodic intervals until a significant titer of anti-ET-NANB antibody is detected in the serum. The vaccine is intended to protect against ET-NANB infection.

Particularly preferred are vaccines prepared using proteins expressed by the 406.3-2 and 406.4-2 clones described herein and equivalents thereof, including fragments of the expressed proteins. Since these clones have already been demonstrated to be reactive with a variety of human HEV-positive sera, their utility in protecting against a variety of HEV strains is indicated.

D. Prophylactic and Therapeutic Antibodies and Antisera

In addition to use as a vaccine, the compositions can be used to prepare antibodies to ET-NANB virus particles. The antibodies can be used directly as antiviral agents. To prepare antibodies, a host animal is immunized using the virus particles or, as appropriate, non-particle antigens native to the virus particle are bound to a carrier as described above for vaccines. The host serum or plasma is collected following an appropriate time interval to provide a composition comprising antibodies reactive with the virus particle. The gamma globulin fraction or the IgG antibodies can be obtained, for example, by use of saturated ammonium sulfate or DEAE Sephadex, or other techniques known to those skilled in the art. The antibodies are substantially free of many of the adverse side effects which may be associated with other anti-viral agents such as drugs.

The antibody compositions can be made even more compatible with the host system by minimizing potential adverse immune system responses. This is accomplished by removing all or a portion of the FC portion of a foreign species antibody or using an antibody of the same species as the host animal, for example, the use of antibodies from human/human hybridomas.

The antibodies can also be used as a means of enhancing the immune response since antibody-virus complexes are recognized by macrophages. The antibodies can be administered in amounts similar to those used for other therapeutic administrations of antibody. For example, pooled gamma globulin is administered at 0.02-0.1 ml/lb body weight during the early incubation of other viral diseases such as rabies, measles and hepatitis B to interfere with viral entry into cells. Thus, antibodies reactive with the ET-NANB virus particle can be passively administered alone or in conjunction with another anti-viral agent to a host infected with an ET-NANB virus to enhance the immune

response and/or the effectiveness of an antiviral drug.

Alternatively, anti-ET-NANB-virus antibodies can be induced by administering anti-idiotype antibodies as immunogens. Conveniently, a purified anti-ET-NANB-virus antibody preparation prepared as described above is used to induce anti-idiotype antibody in a host animal. The composition is administered to the host animal in a suitable diluent. Following administration, usually repeated administration, the host produces anti-idiotype antibody. To eliminate an immunogenic response to the Fc region, antibodies produced by the same species as the host animal can be used or the Fc region of the administered antibodies can be removed. Following induction of anti-idiotype antibody in the host animal, serum or plasma is removed to provide an antibody composition. The composition can be purified as described above for anti-ET-NANB virus antibodies, or by affinity chromatography using anti-ET-NANB-virus antibodies bound to the affinity matrix. The anti-idiotype antibodies produced are similar in conformation to the authentic ET-NANB antigen and may be used to prepare an ET-NANB vaccine rather than using a ET-NANB particle antigen.

When used as a means of inducing anti-ET-NANB virus antibodies in a patient, the manner of injecting the antibody is the same as for vaccination purposes, namely intramuscularly, intraperitoneally, subcutaneously or the like in an effective concentration in a physiologically suitable diluent with or without adjuvant. One or more booster injections may be desirable. The anti-idiotype method of induction of anti-ET-NANB virus antibodies can alleviate problems which may be caused by passive administration of anti-ET-NANB-virus antibodies, such as an adverse immune response, and those associated

with administration of purified blood components, such as infection with as yet undiscovered viruses.

The ET-NANB derived proteins of the invention are also intended for use in producing antiserum designed for pre- or post-exposure prophylaxis. Here an ET-NANB protein, or mixture of proteins is formulated with a suitable adjuvant and administered by injection to human volunteers, according to known methods for producing human antisera. Antibody response to the injected proteins is monitored, during a several- week period following immunization, by periodic serum sampling to detect the presence an anti-ET-NANB serum antibodies, as described in Section IIA above.

The antiserum from immunized individuals may be administered as a pre-exposure prophylactic measure for individuals who are at risk of contracting infection. The antiserum is also useful in treating an individual post-exposure, analogous to the use of high titer antiserum against hepatitis B virus for post-exposure prophylaxis.

E. Monoclonal Antibodies

For both in vivo use of antibodies to ET-NANB virus particles and proteins and anti-idiotype antibodies and diagnostic use, it may be preferable to use monoclonal antibodies. Monoclonal anti-virus particle antibodies or anti-idiotype antibodies can be produced as follows. The spleen or lymphocytes from an immunized animal are removed and immortalized or used to prepare hybridomas by methods known to those skilled in the art. To produce a human-human hybridoma, a human lymphocyte donor is selected. A donor known to be infected with a ET-NANB virus (where infection has been shown for example by the presence of anti-virus antibodies in the blood or by virus culture) may serve as a suitable lymphocyte donor. Lymphocytes can be isolated from a peripheral blood sample or spleen cells may be used if the donor is

subject to splenectomy. Epstein-Barr virus (EBV) can be used to immortalize human lymphocytes or a human fusion partner can be used to produce human-human hybridomas. Primary in vitro immunization with peptides can also be used in the generation of human monoclonal antibodies.

Antibodies secreted by the immortalized cells are screened to determine the clones that secrete antibodies of the desired specificity. For monoclonal anti-virus particle antibodies, the antibodies must bind to ET-NANB virus particles. For monoclonal anti-idiotype antibodies, the antibodies must bind to anti-virus particle antibodies. Cells producing antibodies of the desired specificity are selected.

The following examples illustrate various aspects of the invention, but are in no way intended to limit the scope thereof.

Material

The materials used in the following Examples were as follows:

Enzymes: DNAse I and alkaline phosphatase were obtained from Boehringer Mannheim Biochemicals (BMB, Indianapolis, IN); EcoRI, EcoRI methylase, DNA ligase, and DNA Polymerase I, from New England Biolabs (NEB, Beverly MA); and RNase A was obtained from Sigma (St. Louis, MO) .

Other reagents: EcoRI linkers were obtained from NEB; and nitro blue tetrazolium (NBT), S-bromo-4-chloro-3-indolyl phosphate (BCIP) S-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (Xgal) and isopropyl B-D-thiogalactopyranoside (IPTG) were obtained from Sigma.

cDNA synthesis kit and random priming labeling kits are available from Boehringer-Mannheim Biochemical (BMB, Indianapolis, IN).

Example 1

Preparing cDNA Library

A. Source of ET-NANB virus

Two cynomolgus monkeys (cynos) were intravenously injected with a 10% suspension of a stool pool obtained from a second-passage cyno (cyno #37) infected with a strain of ET-NANB virus isolated from Burma cases whose stools were positive for ET-NANB, as evidenced by binding of 27-34 nm virus-like particles (VLPs) in the stool to immune serum from a known ETNANB patient. The animals developed elevated levels of alanine aminotransferase (ALT) between 24-36 days after inoculation, and one excreted 27-34 nm VLPs in its bile in the pre-acute phase of infection.

The bile duct of each infected animal was cannulated and about 1-3 cc of bile was collected daily. RNA was extracted from one bile specimen (cyno #121) by hot phenol extraction, using a standard RNA isolation procedure. Double-strand cDNA was formed from the isolated RNA by a random primer for first-strand generation, using a cDNA synthesis kit obtained from Boehringer-Mannheim (Indianapolis, IN).

B. Cloning the Duplex Fragments

The duplex cDNA fragments were blunt-ended with T4 DNA polymerase under standard conditions (Maniatis, p. 118), then extracted with phenol/chloroform and precipitated with ethanol. The blunt-ended material was ligated with EcoRI linkers under standard conditions (Maniatis, pp. 396-397) and digested with EcoRI to remove redundant linker ends. Non-ligated linkers were removed by sequential isopropanol precipitation.

Lambda gt10 phage vector (Huynh) was obtained from Promega Biotec (Madison, WI). This cloning vector has a unique EcoRI cloning site in the phage CI repressor gene. The cDNA fragments from above were introduced into the EcoRI site by mixing 0.5 -

1.0 µg EcoRI-cleaved gt10, 0.5-3 µl of the above duplex fragments, 0.5 µl 10X ligation buffer, 0.5 µl ligase (200 units), and distilled water to 5 µl. The mixture was incubated overnight at 14°C, followed by in vitro packaging, according to standard methods (Maniatis, pp. 256-268).

The packaged phage were used to infect an E. coli hfl strain, such as strain HG415. Alternatively, E. coli, strain C600 hfl available from Promega Biotec, Madison, WI, could be used. The percentage of recombinant plaques obtained with insertion of the EcoRI-ended fragments was less than 5% by analysis of 20 random plaques.

The resultant cDNA library was plated and phage were eluted from the selection plates by addition of elution buffer. After DNA extraction from the phage, the DNA was digested with EcoRI to release the heterogeneous insert population, and the DNA fragments were fractionated on agarose to remove phage fragments. The 500-4,000 basepair inserts were isolated and recloned into lambda gt10 as above, and the packaged phage was used to infect E. coli strain HG415. The percentage of successful recombinants was greater than 95%. The phage library was plated on E. coli strain HG415, at about 5,000 plaques/plate, on a total of 8 plates.

Example 2

Selecting ET-NANB Cloned Fragments

A. cDNA Probes

Duplex cDNA fragments from noninfected and ETNANB-infected cynomolgus monkeys were prepared as in Example 1. The cDNA fragments were radiolabeled by random priming, using a random-priming labeling kit obtained from Boehringer-Mannheim (Indianapolis, IN).

B. Clone Selection

The plated cDNA library from Example 1 was transferred to each of two nitrocellulose filters, and the phage DNA was fixed on the filters by baking, according to standard methods (Maniatis, pp. 320323). The duplicate filters were hybridized with either infected-source or control CDNA probes from above. Autoradiographs of the filters were examined to identify library clones which hybridized with radiolabeled CDNA probes from infected source only, i.e., did not hybridize with cDNA probes from the non-infected source. Sixteen such clones, out of a total of about 40,000 clones examined, were identified by this subtraction selection method.

Each of the sixteen clones was picked and replated at low concentration on an agar plate. The clones on each plate were transferred to two nitrocellulose ag duplicate lifts, and examined for hybridization to radiolabeled cDNA probes from infected and noninfected sources, as above. Clones were selected which showed selective binding for infected-source probes (i.e., binding with infected-source probes and substantially no binding with non-infected-source probes). One of the clones which bound selectively to probe from infected source was isolated for further study. The selected vector was identified as lambda gt10-1.1, indicated in Figure 1.

Example 3

ET-NANB Sequence

Clone lambda gt10-1.1 from Example 2 was digested with EcoRI to release the heterologous insert, which was separated from the vector fragments by gel electrophoresis. The electrophoretic mobility of the fragment was consistent with a 1.33 kb fragment. This fragment, which contained EcoRI ends, was inserted into the EcoRI site of a pTZKF1 vector, whose construction and properties are described in co-owned U.S. patent application for "Cloning Vector System and

Method for Rare Clone Identification", Serial No. 125, 650, filed November 25, 1987. Briefly, and as illustrated in Figure 1, this plasmid contains a unique EcoRI site adjacent a T7 polymerase promoter site, and plasmid and phage origins of replication. The sequence immediately adjacent each side of the EcoRI site is known. E. coli BB4 bacteria, obtained from Stratagene (La Jolla, CA, were transformed with the plasmid.

Radiolabeled ET-NANB probe was prepared by excising the 1.33 kb insert from the lambda gt10-1.1 phage in Example 2, separating the fragment by gel electrophoresis, and randomly labeling as above. Bacteria transfected with the above pTZKF1 and containing the desired ET-NANB insert were selected by replica lift and hybridization with the radiolabeled ET-NANB probe, according to methods outlined in Example 2.

One bacterial colony containing a successful recombinant was used for sequencing a portion of the 1.33 kb insert. This isolate, designated pTZKF1(ET1.1), has been deposited with the American Type Culture Collection, and is identified by ATCC deposit no. 67717. Using a standard dideoxy sequencing procedure, and primers for the sequences flanking the EcoRI site, about 200-250 basepairs of sequence from the 5'-end region and 3'-end region of the insert were obtained. The sequences are given above in Section II. Later sequencing by the same techniques gave the full sequence in both directions, also given above.

Example 4

Detecting ET-NANB Sequences

cDNA fragment mixtures from the bile of noninfected and ET-NANB-infected cynomolgus monkeys were prepared as above. The cDNA fragments obtained from human stool samples were prepared as follows.

Thi[redacted] ml of a 10% stool suspension obtained from an individual from Mexico diagnosed as infected with ET-NANB as a result of an ET-NANB outbreak, and a similar volume of stool from a healthy, non-infected individual, were layered over a 10% sucrose density gradient cushion, and centrifuged at 25,000 x g for 6 hr in an SW27 rotor, at 15°C. The pelleted material from the infected-source stool contained 27-34 nm VLP particles characteristic of ET-NANB infection in the infected-stool sample. RNA was isolated from the sucrose-gradient pellets in both the infected and non-infected samples, and the isolated RNA was used to produce cDNA fragments as described in Example 1.

The CDNA fragment mixtures from infected and non-infected bile source, and from infected and non-infected human-stool source were each amplified by a novel linker/primer replication method described in co-owned patent application serial number 07/208,512 for "DNA Amplification and Subtraction Technique," filed June 17, 1988. Briefly, the fragments in each sample were blunt-ended with DNA Pol I then extracted with phenol/chloroform and precipitated with ethanol. The blunt-ended material was ligated with linkers having the following sequence (top or 5' sequence has SEQ ID NO.21; bottom or 3'sequence has SEQ ID NO:22):

5'-GGAATTCGCGGCCGCTCG-3'

3'-TTCCTTAAGCGCCGGCGAGC-5'

The duplex fragments were digested with NruI to remove linker dimers, mixed with a primer having the sequence 5'-GGAATTCGCGGCCGCTCG-3', and then heat denatured and cooled to room temperature to form single-strand DNA/primer complexes. The complexes were replicated to form duplex fragments by addition of Thermus aquaticus ('Taq') polymerase and all four deoxynucleotides. The replication procedures, involving successive strand denaturation, formation of

strand/primer complexes, and replication, was repeated 25 times.

The amplified cDNA sequences were fractionated by agarose gel electrophoresis, using a 2% agarose matrix. After transfer of the DNA fragments from the agarose gels to nitrocellulose paper, the filters were hybridized to a random-labeled 32p probe prepared by (i) treating the pTZKF1(ET1.1) plasmid from above with EcoRI, (ii) isolating the released 1.33 kb ET-NANB fragment, and (iii) randomly labeling the isolated fragment. The probe hybridization wag performed by conventional Southern blotting methods (Maniatis, pp. 382-389). Figure 2 shows the hybridization pattern obtained with cDNAs from infected (I) and non-infected (N) bile sources (2A) and from infected (I) and noninfected (N) human stool sources (2B). As seen, the ET-NANB probe hybridized with fragments obtained from both of the infected sources, but was non-homologous to sequences obtained from either of the non-infected sources, thus confirming the specificity of derived sequence.

Southern blots of the radiolabeled 1.33 kb fragment with genomic DNA fragments from both human and cynomolgus-monkey DNA were also prepared. No probe hybridization to either of the genomic fragment mixtures was observed, confirming that the ET-NANB sequence is exogenous to either human or cynomolgus genome.

Example 5

Expressing ET-NANB Proteins

A. Preparing ET-NANB Coding Sequences

The pTZKF1(ET1.1) plasmid from Example 2 was digested with EcoRI to release the 1.33 kb ET-NANB insert which was purified from the linearized plasmid by gel electrophoresis. The purified fragment was suspended in a standard digest buffer (0.5M Tris HCl, pH 7.5; 1 mg/ml BSA; 10mM MnC12) to a concentration of

about 1 mg/ml and digested with DNAse I at room temperature for about 5 minutes. These reaction conditions were determined from a prior calibration study, in which the incubation time required to produce predominantly 100-300 basepair fragments was determined. The material was extracted with phenol/chloroform before ethanol precipitation.

The fragments in the digest mixture were blunt-ended and ligated with EcoRI linkers as in Example 1. The resultant fragments were analyzed by electrophoresis (5-10V/cm) on 1.2% agarose gel, using PhiX174/HaeIII and lambda/HindIII size markers. The 100-300 bp fraction was eluted onto NA45 strips (Schleicher and Schuell), which were then placed into 1.5 ml microtubes with eluting solution (1 M NaCl, 50 mM arginine, pH 9.0), and incubated at 67°C for 30-60 minutes. The eluted DNA was phenol/chloroform extracted and then precipitated with two volumes of ethanol. The pellet was resuspended in 20 μl TE (0.01 M Tris HCl, pH 7.5, 0.001 M EDTA).

B. Cloning in an Expression Vector

Lambda gt11 phage vector (Huynh) was obtained from Promega Biotec (Madison, WI). This cloning vector has a unique EcoRI cloning site 53 base pairs upstream from the beta-galactosidase translation termination codon. The genomic fragments from above, provided either directly from coding sequences (Example 5) or after amplification of cDNA (Example 4), were introduced into the EcoRI site by mixing 0.5-1.0 μg EcoRI-cleaved gt11, 0.3-3 μl of the above sized fragments, 0.5 μl 10X ligation buffer (above), 0.5 μl ligase (200 units), and distilled water to 5 μl. The mixture was incubated overnight at 14°C, followed by *in vitro* packaging, according to standard methods (Maniatis, pp. 256-268).

The packaged phage were used to infect *E. coli* strain KM392, obtained from Dr. Kevin Moore, DNAX

(Palo Alto, CA). Alternatively, E. Coli strain Y1090, available from the American Type Culture Collection (ATCC #37197), could be used. The infected bacteria were plated and the resultant colonies were checked for loss of beta-galactosidase activity-(clear plaques) in the presence of X-gal using a standard X-gal substrate plaque assay method (Maniatis). About 50% of the phage plaques showed loss of beta-galactosidase enzyme activity (recombinants).

C. Screening for ET-NANB Recombinant Proteins

ET-NANB convalescent antiserum was obtained from patients infected during documented ET-NANB outbreaks in Mexico, Borneo, Pakistan, Somalia, and Burma. The sera were immunoreactive with VLPs in stool specimens from each of several other patients with ET-NANB hepatitis.

A lawn of E. coli KM392 cells infected with about 104 pfu of the phage stock from above was prepared on a 150 mm plate and incubated, inverted, for 5-8 hours at 37°C. The lawn was overlaid with a nitrocellulose sheet, causing transfer of expressed ETNANB recombinant protein from the plaques to the paper. The plate and filter were indexed for matching corresponding plate and filter positions.

The filter was washed twice in TBST buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20), blocked with AIB (TBST buffer with 1% gelatin), washed again in TBST, and incubated overnight after addition of antiserum (diluted to 1:50 in AIB, 12-15 ml/plate). The sheet was washed twice in TBST and then contacted with enzyme-labeled anti-human antibody to attach the labeled antibody at filter sites containing antigen recognized by the antiserum. After a final washing, the filter was developed in a substrate medium containing 33 µl NBT (50 mg/ml stock solution maintained at 4°C) mixed with 16 µl BCIP (50 mg/ml stock solution maintained at 4°C) in 5 ml of alkaline

phosphatase buffer (100 mM Tris, 9.5, 100 mM NaCl, 5 mM MgC12). Purple color appeared at points of antigen production, as recognized by the antiserum.

D. Screening Plating

The areas of antigen production determined in the previous step were replated at about 100-200 pfu on an 82 mm plate. The above steps, beginning with a 5-8 hour incubation, through NBT-BCIP development, were repeated in order to plaque purify phage secreting an antigen capable of reacting with the ET-NANB antibody. The identified plaques were picked and eluted in phage buffer (Maniatis, p. 443).

E. Epitope Identification

A series of subclones derived from the original pTZKF1 (ET1.1) plasmid from Example 2 were isolated using the same techniques described above. Each of these five subclones were immunoreactive with a pool of anti-ET antisera noted in C. The subclones contained short sequences from the "reverse" sequence set forth previously. The beginning and ending points of the sequences in the subclones (relative to the full "reverse" sequence), are identified in the table below.

TABLE 1

Subclone	Position in "Reverse" Sequence	
	5'-end	3'-end
Y1	522	643
Y2	594	667
Y3	508	665
Y4	558	752
Y5	545	665

Since all of the gene sequences identified in the table must contain the coding sequence for the epitope, it is apparent that the coding sequence for the epitope falls in the region between nucleotide 594 (5'-end) and 643 (3'-end). Genetic sequences equivalent to and complementary to this relatively short sequence are therefore particularly preferred aspects of the present invention, as are peptides produced using this coding region.

A second series of clones identifying an altogether different epitope was isolated with only Mexican serum.

TABLE 2

Subclone	Position in "Forward" Sequence	
	5'end	3' end
ET 2-2	2	193
ET 8-3	2	135
ET 9-1	2	109
ET 13-1	2	101

The coding system for this epitope falls between nucleotide 2 (S -end) and 101 (3 -end). Genetic sequences related to this short sequence are therefore also preferred, as are peptides produced using this coding region.

Two particularly preferred subclones for use in preparing polypeptides containing epitopes specific for HEV are the 406.3-2 and 406.4-2 clones whose sequences are set forth above. These sequences were isolated from an amplified cDNA library derived from a Mexican stool. Using the techniques described in this section, polypeptides expressed by these clones have been tested for immunoreactivity against a number of different human HEV-positive sera obtained from sources around the world. As shown in Table 3 below, 8 sera immunoreactive with the polypeptide expressed by the 406.4-2, and 6 sera immunoreacted with polypeptide expressed by the 406.3-2 clone.

For comparison, the Table also shows reactivity of the various human sera with the Y2 clone identified in Table 1 above. Only one of the sera reacted with the polypeptide expressed by this clone. No immunoreactivity was seen for normal expression products of the gt11 vector.

Table 3

Immunoreactivity of HEV Recombinant Proteins: Human Sera

Sera	Source	Stage[1]	406.3-2	406.4-2	Y2	λgt11
FVH-21	Burma	A	-	-	-	-
FVH-8	Burma	A	-	+	+	-
SOM-19	Somalia	A	+	+	-	-
SOM-20	Somalia	A	+	+	-	-
IM-35	Borneo	A	+	+	-	-
IM-36	Borneo	A	-	-	-	-
PAK-1	Pakistan	A	+	+	-	-
FFI-4	Mexico	A	+	+	-	-

FFI-125	Mexico	A	-	+	-	-
F 387 IC	Mexico	C	+	+	ND	-
Normal	U.S.A.	-	-	-	-	-

[1]A = acute; C = convalescent

While the invention has been described with reference to particular embodiments, methods, construction and use, it will be apparent to those skilled in the art that various changes and modifications can be made without departing from the invention.